The influence of zinc on the physiology of industrial strains of *Saccharomyces cerevisiae*

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Abstract

The yeast, *Saccharomyces cerevisiae* requires certain elements for the growth and development of healthy cultures. The divalent cation, zinc is of paramount importance to this yeast, as zinc is a structurally and functionally essential metal that cannot be replaced by any other element. Zinc accumulation by *S. cerevisiae* is a biphasic response, consisting of a rapid metabolism independent and a metabolism dependent phase. Metabolism-independent metal ion accumulation is a physical process, whereby the ions are associated with the cell wall. This stage of uptake is often referred to as biosorption and zinc uptake is influenced by temperature, pH, biomass concentration and the presence of competing ions. The second phase of zinc uptake (metabolism-dependent metal ion accumulation) concerns the intracellular accumulation of the ions. This biological accumulation, often abbreviated to bioaccumulation, is slower than biosorption as the zinc ions are transported into the cell, via the plasma membrane by the energy consuming process, active transport. The presence and type of metabolisable energy source, metabolic inhibitors, as well as the factors that affect biosorption also affect bioaccumulation. The genetics governing zinc accumulation by *S. cerevisiae* has recently been unravelled (Zhao and Eide, 1996a & b). Research has shown that a high (*ZRT1*) and a low (*ZRT2*) affinity transporter proteins exists, which act in zinc limiting and zinc replete conditions, respectively. Once the transporters aid zinc uptake into the cell, this important divalent cation is either utilised immediately or compartmentalised in the vacuole until required. Zinc accumulation is influenced by yeast cell physiology. Upon examination of zinc uptake with respect to cell growth, in various metabolisable energy sources, the results demonstrate that zinc is influential in the growth of industrial relevant strains of *S. cerevisiae*, and that zinc accumulation is affected by the presence and type of metabolisable energy source *e.g.* glucose, fructose, maltose and sucrose. Optimal growth was achieved when the lager yeast and wine yeast was grown in a minimal media containing sucrose as the metabolisable energy source after a 24 hour period with distillers yeast and bakers yeast growth was maximum when grown for 24 hours in a fructose supplemented media. The industrial strains of yeast studied appeared to sequester maximum zinc when the YPDM was supplemented with

monosaccharides, as opposed to disaccharide, after a 24 hour examination period. The accumulation of zinc by *S. cerevisiae* lager yeast is a cyclical event with uptake occurring during lag and early exponential phase of growth, with zinc appearing to convey a protective effect on cells which have been subjected to a chemical (15% ethanol) and a physical (heat shock- 45°C) stress. The influence of zinc accumulation on yeast cell physiology was studied with respect to specific enzyme (Alcohol Dehydrogenase) and metabolite (ethanol) production. The results demonstrate a general trend, with more ADH produced when the cells have sequestered more zinc, this in turn had a positive effect on the overall ethanol production of a strain of lager yeast.

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General Introduction

1.1 Introduction to industrial strains of yeast

The biotechnological exploitation of industrial strains of yeast is allowing yeast cells to be produced for more diverse functions. The traditionally classed baking and brewing yeast (*Saccharomyces cerevisiae*) can be both beneficial and/or detrimental to human health. The beneficial yeast cell is responsible for the production of alcohol (solvent, potable and gasohol), food and dairy products, pharmaceuticals, enzymes, vitamins, and dietary supplements. Cloned therapeutic gene products from *S. cerevisiae* include viral based products (*e.g.* hepatitis B surface antigen), hormones (insulin), antibodies, interferons, blood proteins and related products (fibrinogen). The production of these products by GRAS (generally regarded as safe) status yeast cells represents an important factor for the production of food and pharmaceuticals. In the forthcoming years the exploitation of industrial yeasts will continue, with *S. cerevisiae* being an important role model in the study of human diseases *e.g.* cancer, and being involved experimentally in the production of vaccines for human afflictions *e.g.* AIDS. *Saccharomyces spp.* are not the only yeasts to be exploited by industry. The biodiversity within yeast species is being further exploited, and different species are now involved in many biotechnological fields (see Table 1).

Table 1: Examples of Non-*Saccharomyces* yeast exploited biotechnologically (adapted form Walker, 1998a)

Yeast Species	Biotechnological Exploitation
Kluyveromyces marxianus and *K. lactis*	Animal feed yeast biomass from whey lactose. Sources of lactase
Candida utilis	Single cell protein (SCP)
Phaffia rhodozyma	Carotene pigment
Saccharomyces boulardii	Biotherapeutic agent (probiotic)
Pichia pastoris	SCP, recombinant proteins
Yarrowia lipolytica	SCP, recombinant proteins
Rhodotorula glutinis	Single cell oil, as substitute for edible and non-edible oils from cheap carbon sources

1.2 Introduction to *Saccharomyces cerevisiae*

The scientific community has been studying this organism in depth for the past 50 years. This has resulted in an amazing amount of available literature revealing different aspects of the growth, physiology, metabolism, and genetics of this yeast.

For the last few decades *S. cerevisiae* has been used as the model organism in the field of molecular genetics. The use of *S. cerevisiae* in this research field is due to the similarities that exist between the cellular mechanisms of DNA replication, recombination, cell division, and metabolism between yeast cells and higher eukaryotes.

In April 1996, the genome of *S. cerevisiae* was mapped and the resulting information has allowed the exploitation of this organism to proceed further in all areas including genetics, biochemistry and importantly physiology.

Yeasts are heterotrophic, unicellular organisms, which are characterised by a wide dispersion of natural habitats. Yeast cells may reproduce asexually by either budding, like

S. cerevisiae, or by fission, like *Schizosaccharomyces pombe*. Under certain conditions some dimorphic yeast species may grow in a manner that resemble fungi *i.e.* by the production of mycelium *e.g. Candida albicans*. As well as replication through the aforementioned asexual mechanisms, yeast cells may also replicate through a sexual mode of reproduction. For sexual reproduction to commence in *S. cerevisiae*, cells of the opposite mating types (a and α) secrete pheromones, which in turn promote an attraction between the cell types and the cells may initiate sexual reproduction in a process known as conjugation.

The industrial important unicellular organism, *S. cerevisiae* has been classified as a true fungus, and can be taxonomically catalogued as described in Table 2.

Table 2: Taxonomic classification of the yeast *Saccharomyces cerevisiae*.

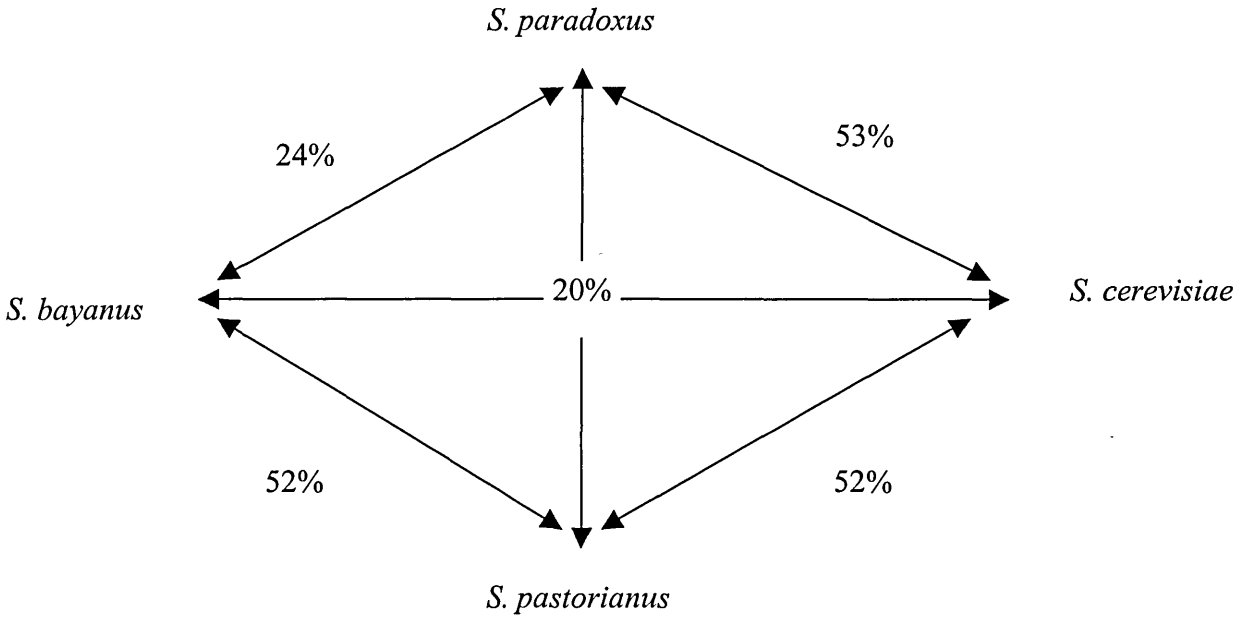
Taxonomic Category	Example
Phylum	*Ascomycotina*
Families	*Saccharomycetaceae*
Subfamilies	*Saccharomycetoideae*
Genera	*Saccharomyces*
Species	*cerevisiae*

The subdivision of yeast into each category is dependent upon the individual characteristics of the yeast with the generic classification using morphological characteristics, and the species determined through biochemical studies, with particular attention paid to the ability

of the yeast to ferment certain sugars. Recently, molecular studies have assisted in taxonomic classification of yeasts *e.g.* karyotyping and DNA fingerprinting.

The taxonomic classification of the *Saccharomyces* genus has over the years been subject to much debate. A recent review of the taxonomic literature referring to the classification of this genus discovered that a published document by Guilliermont (*Les Levures*) in 1912 described 20 species belonging to the *Saccharomyces* genus. However, in 1984 Yarrow published in the "*The Yeasts, A taxonomic study*" that all previously detailed species could be merged and described as one species, which was *S. cerevisiae.* This remained the subject of debate until the advent of molecular techniques, more specifically nDNA-nDNA reassociation. Using this technique only 4 species were found to belong to the genus *Saccharomyces* and are therefore referred to as *Saccharomyces* "sensu stricto". These described species, which are genetically similar are detailed in Figure 1.1, as is the degree of sequence homology (Vaughan-Martini and Martini, 1995).

Figure 1.1: Molecular relationships between the species of the genus *Saccharomyces* "sensu stricto" (adapted from Vaughan-Martini and Martini, 1995).



The industrial yeasts utilised throughout this study were a lager brewers yeast, a distillers yeast, a wine making yeast and a baking yeast. Brewing yeasts may be referred to as either ale or lager yeasts. These brewing yeasts differ with respect to fermentation temperature and tolerance and also in their biochemical ability to utilise melibiose *e.g.* lager yeasts can effectively utilise melibiose, whereas ale yeasts, which do not possess the enzyme melibiase are incapable of utilising this sugar (Stewart and Russell, 1998). This suggests that the genetic background of these yeasts may be different. Ale yeasts are accepted as strains of *S. cerevisiae* whereas brewers generally refer to lager yeasts as *S. cerevisiae var. carlsbergenesis* (Stewart and Russell, 1998). Taxonomists might argue this as incorrect, and that lager yeasts should be more correctly referred to as *S. pastorianus* (Vaughan-Martini and Martini, 1995). Many distilling companies use either a brewers or bakers strain of yeast for fermentation (Stewart and Russell, 1998). Therefore, throughout this thesis all of the industrial yeasts studied will be referred to as strains of *S. cerevisiae.*

1.3 Yeast cell physiology

Yeast cell physiology refers to how yeast cells feed, metabolise, grow, reproduce (either sexually, or asexually), survive and ultimately die (reviewed in Walker, 1998a). For the maintenance of yeast cell growth, particular nutrients must be readily available in either micro or macromolar amounts, and the supply of these nutrients, in appropriate concentrations, will allow for accelerated growth and an increase in the biomass yield (Jones and Greenfield, 1984). Table 3 is a representation of the basic nutritional requirements and the role that these nutrients play in order for the yeast cell to remain viable and vital.

Table 3: Yeast Cell Nutritional Requirements

Element	Common Source	Cellular Function
Carbon	Sugars	Major structural element of yeast cells in combination with hydrogen, oxygen and nitrogen. Catabolism of carbon compounds also provides energy
Hydrogen	Protons from acidic environment	Transmembrane proton motive force vital for yeast nutrition. Intracellular acidic pH (around 5-6) necessary for yeast metabolism
Oxygen	Air, O_2	Substrate for respiratory and other mixed function oxidative enzymes. Essential for ergosterol and unsaturated fatty acid synthesis
Nitrogen	NH_4^+ salts, urea, amino acids	Structurally and functionally as organic amino nitrogen in proteins and enzymes
Phosphorus	Phosphates	Energy transduction, nucleic acid and membrane structure
Potassium	K^+ salts	Ionic balance, enzyme activity
Magnesium	Mg^{2+} salts	Enzyme activity, cell and organelle structure
Sulphur	Sulphates, methionine	Sulphydryl amino acids and vitamins
Calcium	Ca^{2+} salts	Possible second messenger in signal transduction
Copper	Cupric salts	Redox pigments
Iron	Ferric salts	Haem-proteins, cytochromes
Manganese	Mn^{2+} salts	Enzyme activity
Zinc	Zn^{2+} salts	Multifunctional, enzyme activity, structural support
Nickel	Ni^{2+} salts	Urease activity
Molybdenum	Na_2MoO_4	Nitrate metabolism, Vitamin B_{12}

(Table adapted from Walker, 1998a)

1.4 Yeast cell growth

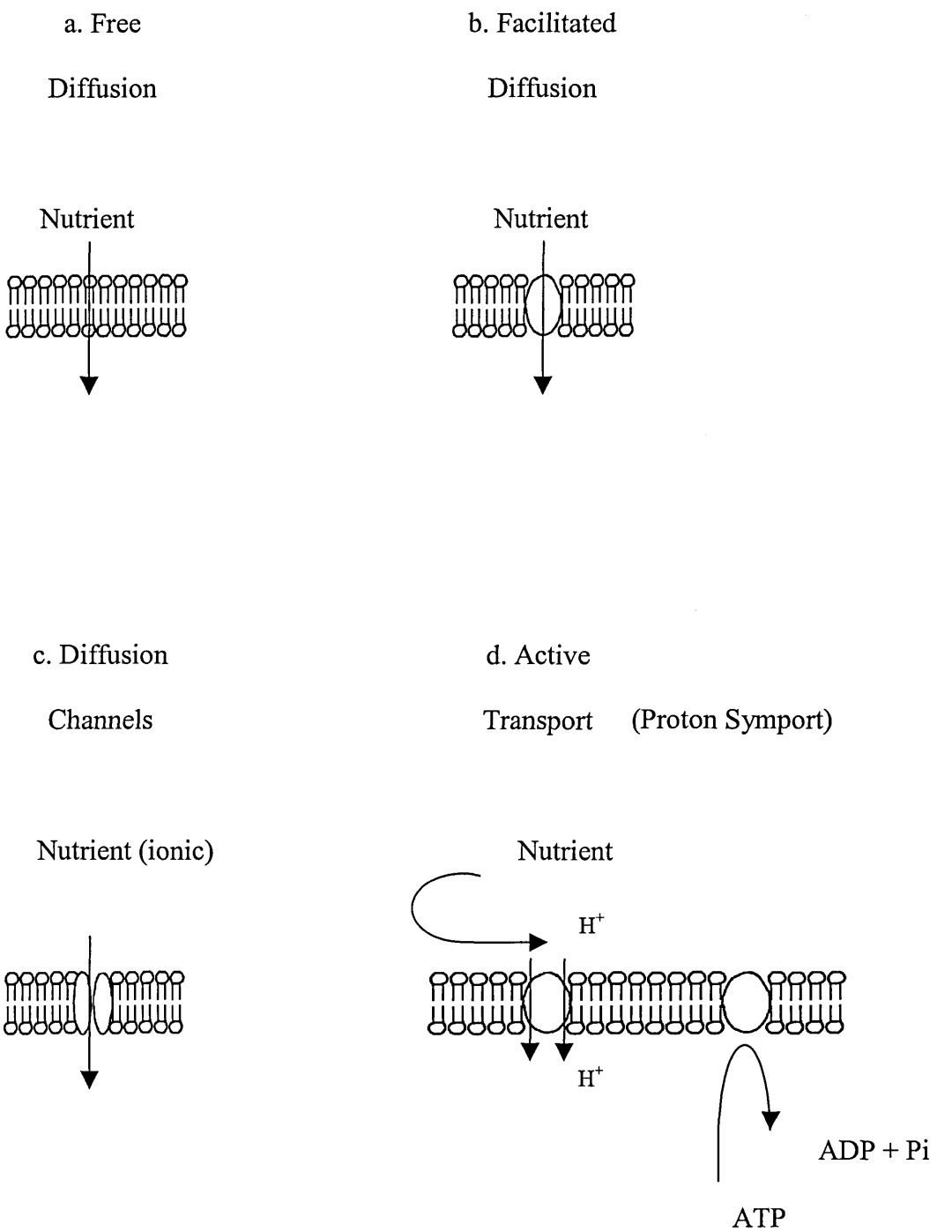
Yeast cell growth is a well-studied concept, which details how cells grow in different environments. Yeast cell growth is concerned with how yeast cells transport and assimilate important nutrients, like carbon compounds, nitrogen compounds, hydrogen and ionic components, as well as trace elements and vitamins (these nutrients are detailed in Table3). A batch fermentation process will allow the yeast cell to enter the growth cycle following lag, exponential, stationary and death (or decline) stages. The biomass will continue to grow, once cells adapt to their new environment, until nutrients become limiting, and/ or the yeast cells are overcome by the production of waste products. The individual stages of growth follow the cell division cycle. The cell division cycle in *S. cerevisiae* consists of a series of events that can proceed independently. However, the stages are interconnected at some points within the cycle. The key events in the cell division cycle are; cell wall growth, cytoplasmic events and DNA synthesis. The cycle begins when the cells reach a critical size, at a stage named "start" and follows the cycle through the key stages of G1, S, G2 and M, resulting in the production of a daughter cell.

1.5 Yeast nutrition and membrane transport

The mechanisms, which have evolved, allowing a yeast cell to feed are termed yeast nutrition. Yeast nutrition refers to how a yeast cell translocates water as well as organic and inorganic nutrients from the surrounding environment into the intracellular matrix, via the cell wall and the plasma membrane resulting in macromolecular biosynthesis and cell growth. The yeast cell wall is often described as a freely porous structure. This is not entirely true (Scherrer *et. al.*, 1974; Klis, 1994,). The *S. cerevisiae* cell wall will allow molecules of a molecular weight less than 300 Da to pass through, and retain any molecules of a weight exceeding 700 Da (Scherrer *et.al.* 1974). Therefore, any molecule that is

capable of passing through the cell wall will find that the plasma membrane is the first obstacle. The plasma membrane is a selectively permeable structure that isolates the cell from its environment. The plasma membrane dictates not only those molecules that enter the cell, but also which metabolites leave. For the orderly entrance of molecules into the cytosol and also the extrusion of such molecules, certain membrane transport processes must be operational. A schematic representation of membrane transport procedures is depicted in Figure 1.2. This diagram demonstrates that nutrients may be transported across the plasma membrane by free diffusion, facilitated diffusion, diffusion channels or active transport.

Figure1.2: Schematic representation of the mechanisms of nutrient transport across the plasma membrane in *S. cerevisiae* (adapted from Walker, 1998a).



Free diffusion (Figure 1.2 a) of nutrients into the yeast cell occurs by passive penetration of the membrane bilayer by lipid soluble nutrients. In the case of charged solutes free diffusion is driven by the membrane potential. Free diffusion of nutrients into the yeast cell, is a slow process, and no energy input is required. The secretion of the major fermentation metabolite, ethanol, occurs by free diffusion. During facilitated diffusion (Figure 1.2 b), nutrient uptake is driven by the concentration gradient via a carrier, this process is relatively fast, occurring in a matter of seconds. It is by facilitated diffusion that the monosaccharides glucose and fructose are transported into the cell. Nutrients may also pass directly into the cells via a diffusion channel (Figure 1.2 c). These channels are voltage dependent proteins, which are activated by membrane depolarisation to influx specific ions. The uptake of certain ionic nutrients (*e.g.* K^+) in this fashion is extremely fast, occurring in pico-seconds. The last manner in which nutrients can enter the yeast cell is by active transport (Figure 1.2 d). Uptake by active transport is driven both by a proton motive force and by the nutrient concentration gradient. Active transport can uptake nutrients with protons as in symport (depicted in Figure 1.2 d) or against protons as in antiport mechanisms. These mechanisms can either transport the nutrient into the cytosol with ions (symport) or expel (antiport) ions. Active transport is a slow process, which involves energy expenditure. However, although an energy consumption process, most nutrients (*e.g.* maltose, amino acids, anions and cations) enter the cytosol via active transport.

1.5.1 Carbohydrate transport and utilisation

Sugars represent the main source of carbon and energy that are required by the cell in order to function and proliferate efficiently (Fiechter and Seghezzi, 1992). In 1837, Schwann determined that sugars were the food of yeasts (cited by Barnett, 1997). The yeast *S. cerevisiae* is capable of utilising a wide range of sugars as common "food" sources. These

include monosaccharides (glucose, fructose, mannose, and galactose), disaccharides (maltose and sucrose) and oligosaccharides (maltotriose), as well as alcohols (ethanol), polyols (glycerol) and organic acids (acetate, citrate and succinate)(Vaughan-Martini and Martini, 1998). Although *S. cerevisiae* can assimilate different carbon sources, glucose is the preferred carbohydrate (Lagunas, 1993). Sugars may pass freely through the cell wall. However they cannot permeate the yeast cell plasma membrane (Lagunas, 1993). It was, therefore, thought that other mechanisms must exist in order to transport sugar molecules into the yeast cell for their further utilisation in carbon metabolism.

1.5.2 Transport and utilisation of monosaccharides into the yeast cell

Cirillo (cited by Barnett, 1997) first established the transport of carbohydrates into the yeast cell in 1961. This research showed that monosaccharides entered bakers yeast by facilitated diffusion. Further details of the transport mechanisms have become available over the years, which culminated with the unravelling of the yeast genome. This project acknowledged the presence of at least 20 hexose transporters. These hexose transporters differ in their abundance and also in their affinities for hexose based carbohydrates (Kruckeberg, 1996). Transporters, which may also be referred to as either carriers or permeases, show specific binding for their substrate. Transporters mediate two types of transport in yeast cells: facilitated diffusion and active transport.

The monosaccharide glucose is transported by facilitated diffusion (as depicted in Figure 1.2 b). This is not an energy consuming process and will continue until the glucose concentration has reached equilibrium. Glucose uptake into the cell is genetically controlled and the genes responsible are constitutively expressed (Dickinson, 1999). The genes that control glucose uptake into the yeast cell are the *HXK* genes. These genes

encode the hexose kinase transporters, of which there is speculated to be a high affinity and a low affinity organisation (Dickinson, 1999). This will, therefore, allow for the uptake of glucose in both glucose limiting and glucose replete conditions.

Once the permeases have aided transport of glucose across the plasma membrane and into the cell, the glucose can participate in energy yielding processes. Other hexose-based sugars can also be transported into the cell aided by the glucose transport system. These carbohydrates include fructose, mannose, xylose, and the glucose analogues: 2-deoxyglucose and 6-deoxyglucose (Gancedo and Serrano, 1989).

1.5.3 Transport and utilisation of disaccharides into the yeast cell

Two transporters have been identified in the yeast cell for the transport of disaccharides. These are concerned with the transport of maltose and α-methylglucoside. The disaccharide maltose is a dimer of glucose joined by a α1-4 glycosidic bond. This disaccharide is extremely important in the beverage fermentation industry as it occurs in malt following barley germination and drying, and during the starch hydrolysis stage of mashing. For maltose uptake into the yeast cell a maltose transport system must be operational. This system which actively transports maltose into the cytosol is induced by maltose, but repressed by glucose (Carlson, 1987). The ability of the yeast to use maltose is dependent upon the *MAL* genes. These genes are organised as five independent loci, which are located on different chromosomes (Dickinson, 1999). Each of the active loci includes three genes: a maltase, a maltose permease and a positive regulatory protein (Lagunas, 1993). The permease is a proton-symporter, that is dependent upon the electrochemical gradient (as depicted in Figure 1.2 d) (Carlson, 1987). The transport of one molecule of maltose is co-transported with one proton. Therefore, for the cell to retain electroneutrality it must extrude potassium ions (Lagunas, 1993).

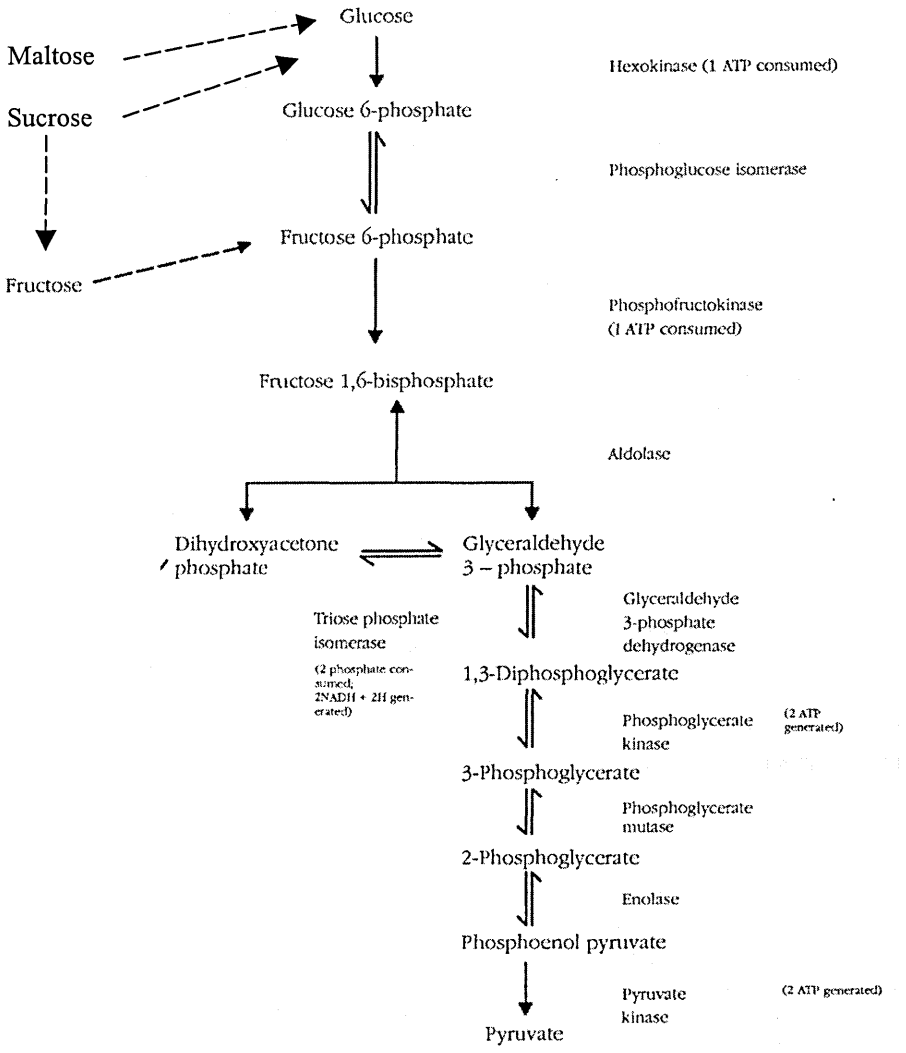
Another disaccharide of interest in this research is sucrose. Sucrose is composed of a glucose and a fructose monomer, which is linked via a α1-4 glycosidic linkage. The utilisation of sucrose by *S. cerevisiae* is dependent upon the expression of one of the *SUC* genes. There are a total of six *SUC* genes (*SUC*1-5 and *SUC*7), and the regulation of these genes is thought to be a complex process. The *SUC* genes encode for invertase (β-D-fructosidase) (Dickinson, 1999). It is disputed that *S. cerevisiae* can produce two forms of invertase. The accepted form, which is known to be a glycoprotein is secreted and is, therefore, responsible for the extracellular hydrolysis of sucrose into glucose and fructose. The disputed form is said to be cytoplasmic and responsible for the internal hydrolysis of sucrose into its component monomers (Mwesigye and Barford, 1994; 1996). The individual components of glucose and fructose can then be utilised within the cell in energy yielding metabolic pathways.

1.6 Carbohydrate utilisation

Carbohydrate utilisation and transport is stringently regulated and metabolically controlled. However, the main metabolic pathways, which are concerned with carbohydrate consumption are glycolysis, the tricarboxylic acid (TCA) cycle, the electron transfer chain, and also the pyruvate dehydrogenase bypass pathway. Glycolysis is concerned with the controlled catabolism of one molecule of glucose, which in turn yields two molecules of pyruvate and ATP, respectively (see Figure 1.2). *S. cerevisiae* can switch its mode of metabolism from fermentation to respiration depending on the availability of oxygen and the concentration of glucose in the media. This switch occurs when the glucose concentration in the media falls below 0.2% (Dickinson. 1999). This is when the Crabtree effect (repression of respiration by glucose) is alleviated.

Fructose is also metabolised through the aforementioned pathways and fits into the glycolytic pathway at the fructose-6-phosphate stage. Maltose (which is broken down into two glucose subunits) and sucrose (which is broken down into the individual monomers of glucose and fructose) are also utilised by the yeast cell through those pathways. Figure 1.3 is a flow diagram of the glycolytic pathway, demonstrating the entry points of the aforementioned sugars. These sugars are either transported into the cell as whole molecules or split into component monomers.

Figure1.3: Schematic representation of the glycolytic pathway in yeast demonstrating the entry points various sugars.



(adapted from Walker, 1998a)

Therefore, in conclusion, *S. cerevisiae* can transport sugars directly into the cell, unchanged, by facilitated diffusion (*e.g.* glucose and fructose). Some sugars can also enter the cell via active transport and then be subjected to cleavage by specific enzymes (*e.g.* maltose cleaved by maltase). Finally in the case of sucrose, which may be unable to be transferred into the cell as an intact disaccharide, the production of extracellular enzymes act to split the sugar into transportable monosaccharides. Once the carbohydrate has entered the cell it can either participate in anabolic or catabolic reactions, or in the case of certain carbohydrates (*e.g.* glycogen and trehalose) will remain in storage until required.

1.7 Metal ion accumulation and transport

The removal of metal ions from solution by biological entities can occur via numerous methods. Generally, the uptake of metal by yeast is a biphasic process, consisting of a metabolism-independent and a metabolism-dependent stage (Mowll and Gadd, 1983). These processes allow yeast cells to accumulate high levels of metal ions.

1.7.1 Metabolism-independent metal ion accumulation

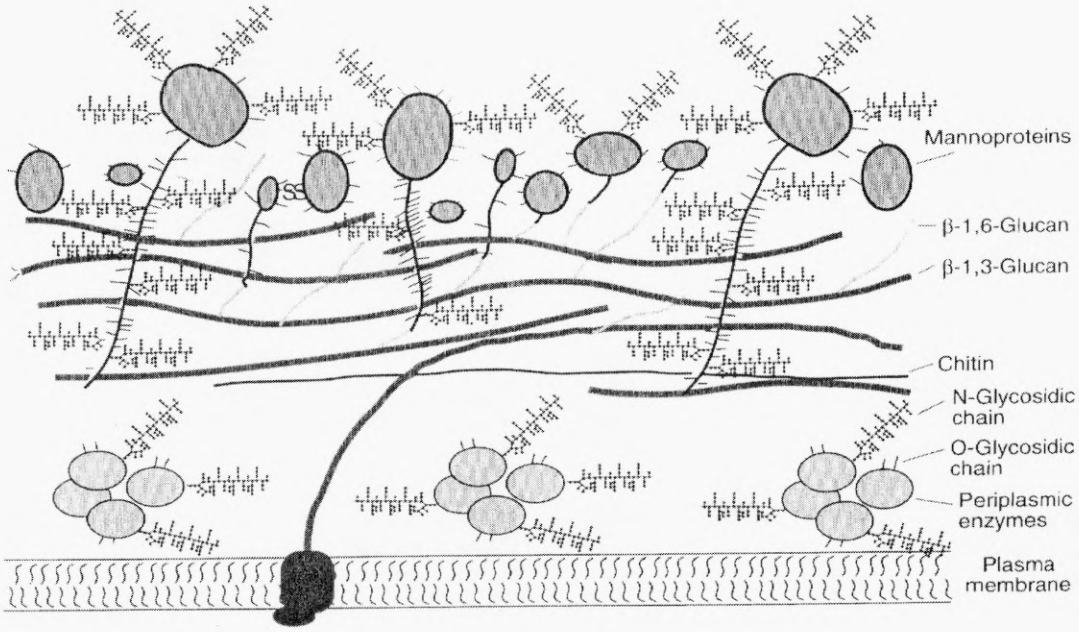
The first stage of the process, metabolism-independent metal ion accumulation, can proceed either alone or in conjunction with metabolism-dependent metal ion accumulation. This first stage is a physical process whereby the metal ions are attracted to the yeast cell wall (Mowll and Gadd, 1983). Metabolism-independent metal uptake can be referred to as adsorption. This adsorption can be of the following types: exchange adsorption, physical/ ideal adsorption or chemical/ activated adsorption (de Rome, 1988). It is extremely difficult to determine between the types. Therefore, any binding of a metal ion to the cell wall/ extracellular material is generally referred to as "biosorption". Biosorption has been defined as "the property of certain types of microbial biomass to bind and concentrate heavy

metals" (Volesky, 1992). Biosorption is the primary stage of ion accumulation and is rapid with sequestration occurring within the first few seconds. Biosorption is not dependent on a metabolisable energy source. However, ion uptake by microbial biomass can be affected by temperature, pH, initial metal ion concentration, biomass concentration and the presence of competing ions (Gadd, 1993). Since biosorption is a physical process the cell does not have to be viable to accumulate metal ions.

1.7.2 The *S. cerevisiae* cell wall

The yeast cell wall is one feature that distinguishes yeast cells from other single celled organisms. It also has a very important role to play in the biosorption of metal ions. The yeast cell wall protects the fragile internal structures from the sometimes, harsh environment. The cell wall controls fluxes between the cytoplasm and the surrounding environment and it also determines the shape of the cell (Remacle, 1990). The composition of the yeast cell wall has been extensively studied (for review see Klis, 1994). And these studies have revealed that the cell wall of *S. cerevisiae* is comprised mainly of polysaccharides which can contribute up to 30% of the total dry weight of the cell (Figure 1.4) (Klis, 1994). These polysaccharides comprise approximately 85% of the total composition of the cell wall, with the individual constituents being glucan, mannan, chitin and chitosan. The remainder of the cell wall consists of proteins and lipids, with a small proportion of inorganic metal ions (Brady *et. al*, 1994).

Figure 1.4: The cell wall of *S. cerevisiae*



(adapted from Walker, 1998a)

The yeast cell wall is negatively charged. This charge can be attributed to the phosphodiester and the carboxyl groups of the individual cell wall components (Remacle, 1990). However, it must be noted that the cell wall features will change in response to the growth phase of the organism, the culture conditions encountered and certain environmental parameters.

Biosorption, therefore, may be viewed primarily as a function of "binding of heavy metal cations to chemical functional groups on the yeast cell wall via ionic and co-ordinate bonds" (Brady and Duncan, 1994). Many cations can bind to the yeast cell wall, and different ions will bind with different affinities. However, most cations will bind to specific residues that

are present in the cell wall *e.g.* copper is attracted to the nitrogen molecules of histidine and zinc has an affinity for the sulphydryl groups of cysteine (Brady and Duncan, 1994).

1.7.3 Metabolism-dependent metal ion accumulation

The second phase of cation sequestration is the metabolism-dependent stage, which can also be referred to as bioaccumulation. Due to the hydrophilic nature of certain ions such as zinc, they cannot cross biological membranes using a passive mechanism (Guerinot and Eide, 1999). Zinc transport into the cell must, therefore, be aided. In metabolism-dependent metal ion accumulation, zinc is actively transported from the extracellular environment into the cell via the plasma membrane. This is accomplished by the aid of a plasma membrane proton-pumping ATPase (White and Gadd, 1987; Nies, 1999). Once the metal is inside the cell it may either be utilised immediately or stored in the vacuole until required (Ramsay and Gadd, 1997). Since bioaccumulation is highly dependent upon the metabolic activities of the cell, it can be severely affected by a variety of situations *e.g.* absence of an energy source, presence of metabolic inhibitors, adverse temperatures and pH.

1.7.4 Plasma membrane ATPase

ATP driven ion pumps are used by cells to generate ion gradients at the expense of ATP hydrolysis or to generate ATP at the expense of an ion gradient (Racker, 1976). These pumps are not unique to yeast, and are found in other lower organisms, *e.g.*, various fungi, bacteria, flagellates and plants. The proton pump is the most complex of these ion pumps and requires a minimum of 8 polypeptide chains that are required for functioning (Racker, 1976). The largest component of the proton pump is the ATPase. It is the function of the H^+- ATPase to transform the energy contained in the ATP into the chemio-osmotic energy of a proton transmembrane gradient (de Kerchove d'Exaerde *et. al.,* 1996). It is through the

production of this transmembrane gradient that allows nutrients to pass into the yeast cell. Yeast plasma membrane transporters ATPases are P-type transporter ATPases, which are characterised by the presence of a Phosphorylated catalytic intermediate (de Kerchove d'Exaerde *et. al.,* 1996). In *S. cerevisiae*, H^+- ATPase can comprise up to 50% of the total membrane protein. As well as providing the energy required to drive nutrients and ions into the cell, H^+- ATPase controls cell pH and overall cell growth (Walker, 1998a).

1.7.5 Intracellular metal detoxification methods

Essential metal ions in excess can still evoke toxic responses in many organisms. Zinc was ignored for years by toxicologists due to its apparently "uninteresting characteristics" (Hardman *et. al.*, 1993). However, zinc can still cause toxic effects and in some cases where excess consumption occurs fatalities have been reported. In a mentally disturbed patient, 461 zinc containing coins were consumed resulting in death (Bennett *et. al.*, 1997 cited in Nies, 1999). Therefore, to eliminate any toxic effects, zinc must either be utilised immediately or compartmentalised until required.

Yeast cells have developed certain detoxification procedures for the toxic effects of heavy metals. Detoxification agents include glutathione peptides and metallothioneins. Glutathione has been identified as the detoxification agent of cadmium in *Schizosaccharomyces pombe* (Mehra and Winge, 1991). Metallothioneins are a family of low molecular weight cysteine-rich proteins, which possess metal binding properties (Tohoyama *et. al.*, 1995). Metallothioneins constitute a reservoir of zinc ions that can be redistributed within the cell. Seven zinc atoms are bound to 20 cysteine residues, which form the 2 metal clusters of the metallothionein (Jiang *et.al.*, 1998). Jiang *et.al.*, (1998) have suggested that the transfer of the stored zinc ions back into circulation from the metallothionein clusters is controlled by both the redox and energy states of the cell .

These two proteins enable yeast cells to withstand high levels of metal ions that would normally be inhibitory to the cells. It has been suggested that the presence of glutathione and metallothioneins can also confer heavy metal resistance in other lower eukaryotes *e.g.* *Candida glabrata* (Perego and Howell, 1997).

1.8 Zinc accumulation, transport and utilisation in *S. cerevisiae*

The divalent cation zinc is of immeasurable importance within the *S. cerevisiae* cell due its necessity for the growth and maintenance of healthy cultures. The role of zinc in the yeast cell has been studied for many years, with the importance of the element firstly recognised in the fungus, *Aspergillus niger* in 1869, by Raulin (cited by Faillia, 1977). Although zinc is not the most abundant element in the earth's crust, it is certainly one of the most biologically exploited. Zinc is the only element that is utilised in all 6 enzyme classes *i.e.* oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases as identified by the International Union of Biochemistry (Vallee and Falchuk, 1993). The yeast cell utilises zinc in many ways, involving the divalent cation in the structure and function of enzymes and proteins, in nucleic acid synthesis and cell division. Zinc is known to stabilise ribosomes and membranes, and has also been implicated in the production of secondary metabolites (Ross, 1994).

1.8.1 Utilisation of zinc in *S. cerevisiae*

The role of zinc gene expression

Zinc plays an important role in the synthesis of both enzymes and proteins. These zinc dependent enzymes are all produced either intracellularly or secreted extracellularly (Coleman, 1998).

Zinc influences the production of polymerases, which in turn play a pivotal role in nucleic acid biosynthesis. The cation zinc also acts as a stabilising support for both single and double stranded nucleic acids. This stabilising action is achieved by the tight binding of the zinc to the polynucleotides. This can be attributed to the binding of the positive cation to the negatively charged phosphate groups, which in turn will help to reduce the electrostatic forces which normally caused repulsion within the molecule (Faillia, 1977).

Zinc is also required for gene expression, as zinc ions are a structural component of the zinc finger structural motifs which are found in many transcription factors and zinc ions are a catalytic co-factor for RNA polymerase (Vallee and Falchuk, 1993).

In zinc deficient yeast cultures, protein production is greatly inhibited (Obata *et al.*, 1996) and cell division arrests in the G1 phase of the cell cycle (Walker, 1998a).

1.8.2 Zinc transport into the yeast cell

The fluctuating levels of zinc in the extracellular environment may pose a problem to yeast cells, as physiological responses must be capable of determining both the extracellular and the intracellular zinc concentrations. This nutritional sensing ensures that adequate, but non-toxic, intracellular concentrations of zinc are readily available.

Zinc homeostasis is genetically controlled by the *ZAP1* transcription factor in *S. cerevisiae* (Winge *et. al.*, 1998, Nies, 1999). This autoregulating gene regulates the expression of zinc specific transporters (Eide, 1998). There are two transporters for zinc: *ZRT1* and *ZRT2*. When zinc is limiting in growth media (that is when levels are below 4μM, Zhao and Eide, 1996a) the yeast enters a period of nutrient limitation. In response to this deprivation the genes which encode *ZRT1* are derepressed. These genes code for the high affinity plasma membrane transporter, which becomes operational under zinc limiting conditions (Zhao and Eide, 1996a). It is now known that *ZRT1* is a glycosylated protein that is localised in the

plasma membrane (Guerinot and Eide, 1999). However, in zinc replete conditions, the plasma membrane transporter *ZRT2* controls the movement of zinc into the cell (Zhao and Eide, 1996b). These two plasma membrane transporters are, therefore, responsible for controlling intracellular zinc levels in *S. cerevisiae.* These transporters allow the translocation of zinc ions across the plasma membrane to form an intracellular pool of zinc ions within the cytoplasm. This pool is responsible for supplying zinc to organelles and to the requiring metalloenzymes (MacDiarmid *et. al.* 2000). The amount of zinc that is free in the cytoplasm is minimal (results have demonstrated that the level of cytosolic zinc ranges between 1-5% total zinc, (Simons, 1993; White and Gadd, 1987, respectively). The remainder of the translocated ions are utilised immediately in cellular functions or stored in connection with the vacuole or the metallothioneins.

Although zinc utilisation is controlled at a genetic level, the environmental conditions that yeast cells encounter can also influence the rate of zinc sequestration. For example, low temperature causes the plasma membrane to "cool", thereby altering the structure of the plasma membrane, which will inadvertently affect the total amount of zinc sequestered. pH fluxes can have a detrimental affect on zinc accumulation and may cause alterations in the availability of binding sites within the cell wall.

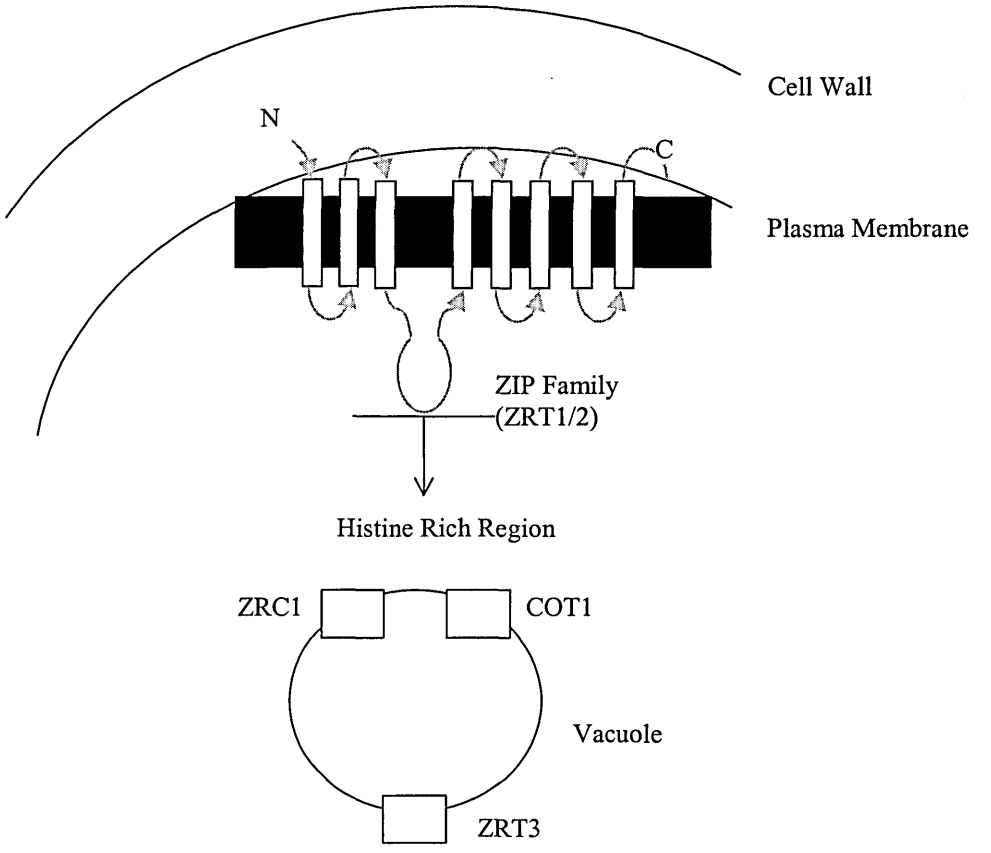
1.8.3 Compartmentalisation of zinc ions into the yeast vacuole

The vacuole is the main storage compartment within the yeast cell, and is responsible for macromolecular degradation, the storage of metabolites and for maintaining cytosolic pH (Ramsay and Gadd, 1997). In addition to previously known vacuolar functions, research has demonstrated a relatively new role for the vacuole. This new role is concerned with the regulation of cytosolic metal ion concentrations (Okorokov *et. al.*, 1980 and 1985; Gharieb and Gadd, 1998; Ramsay and Gadd, 1997). The function of the cytosolic pool of ions is

twofold. Firstly, it is essential for the detoxification of potentially toxic concentrations of metal ions, and secondly, it supplies metal ions, which can participate in cellular functions, if and when necessary. The metal zinc is known to be localised in the vacuole, as is magnesium, manganese, phosphate and potassium (Okorokov *et. al.*, 1980), thereby allowing the cell to withstand high concentrations of these ions. In *S. cerevisiae*, a majority of the intracellular ions are bound to polyphosphate granules, which are located in the vacuole (Yazgan and Őzcengiz, 1994). However, in studies with vacuolar deficient cells, metal ions such as zinc were associated with the cytoplasmic polyphosphates (Ramsay and Gadd, 1997).

The transport of zinc across the vacuolar membrane, like the plasma membrane, is aided by a specific H^+- translocating ATPase (Nies, 1999). This ATPase generates an electrochemical gradient across the membrane by pumping protons, and metal ions, into the vacuole at the same time. In zinc replete cells, zinc is transported into the vacuole via the vacuolar membrane proteins- Zrc 1p and Cot 1p (MacDiarmid *et. al.* 2000). These gene products allow the transport of zinc into the vacuole in an utilisable form. Once the metal ions have been transported into the vacuole they can, if necessary, transport back through the membrane. This re-entry into the cytosol occurs when the zinc concentration in the cytosol decreases and the levels of bioavailable zinc reach a zinc-limiting situation. This decrease in cytosolic zinc promotes the activity of the *ZAP1* transcription factor, which will in turn, increases the expression of *ZRT3* (Guerinot and Eide, 1999). The resultant rise in Zrt 3p activity transports zinc back out of the vacuole and into the cytoplasm. This storage and recycling of zinc ions allows the cell to control cellular zinc and continue to utilise this important divalent cation.

Figure 1.5: Schematic representation of zinc uptake and compartmentalisation within *S. cerevisiae*, with the genes allowing uptake represented (*ZRT1* and *2*; transmembrane zinc transporters, *ZRC1* and *COT1*; control zinc transport into the vacuole and *ZRT3*, controls zinc efflux).



1.8.4 Zinc uptake studies in other organisms

Zinc uptake in *S. cerevisiae* is known to consist of several uptake systems, which are found in the plasma membrane and in the vacuolar membrane. Studies on the ability of other organisms to sequester zinc has also been reported. In the yeasts, *Candida utilis* (Failla *et. al.* 1976) and *C. albicans* (Sabie and Gadd, 1990), in the bacteria, *P. fluorescens* (Di Simine *et. al.* 1998), in numerous crustaceans, (Rainbow, 1995), and also in *Arabidopsis spp.* (Guerinot and Eide, 1999). As research continues in ion uptake systems, the possibility of finding a unicellular hyperaccumulator draws nearer. A hyperaccumulator can be defined as as a biological entity that can accumulate in excess of 10,000μg zinc g^{-1}(Guerinot and Eide, 1999). These studies may help to explain the situation of metal accumulation in higher eukaryotes. The work on zinc accumulation in yeast has helped researchers working on *Arabidopsis.* This plant has the same zinc transporter gene family (*ZIP* family) as *S. cerevisiae.* However, upon examining the DNA sequence database another family member present in *Arabidopsis, ZIP4*, has been found. *ZIP4* is thought to have a chloroplast targeting sequence and is, therefore, absent in yeast (Guerinot and Eide, 1999). The *ZIP* gene family appears to be highly conserved throughout the yeast and plant species examined. Therefore, the possibility of finding a gene family with a high degree of sequence homology to the *ZIP* family in higher eukaryotes, including humans, is likely.

1.9 Practical aspects of zinc uptake by *S. cerevisiae*

The contamination of the environment occurs in all media (air, soil, sediments and water) at both surface and sub-surface levels (Lin *et. al.* 1996). It is due to this reason that contamination by toxic chemicals, including heavy metals, is a growing dilemma. Due to this increasing problem a clean, environmentally friendly way of cleaning up todays pollution is crucial. A possible method of treating chemical waste may be through a

biological remediation process. The objective of bioremediation is the degradation of contaminants to harmless intermediates and end products. A large number of harmful chemicals may be able to be degraded and/or be contained within biological entities, in order that they no longer pose an environmental threat. The use of yeasts to sequester metal cations has been well documented (for review see Blackwell *et. al.* 1995), with astounding results. The studies on the yeast *S. cerevisiae* to accumulate metals and radionuclides (White and Gadd, 1995; White *et. al.*, 1995) have shown that this yeast can accumulate high amounts intracellularly, with compartmentalisation into the vacuole. Metal ion sequestration by yeast is a biphasic process, which can be affected by both physiological and environmental parameters. Metal uptake by *S. cerevisiae* on an industrial scale is possible, and with waste biomass being readily available from several industrial sectors (especially brewing), this makes yeast biomass a cheap and favourable method of cleaning up zinc containing industrial effluents. The use of yeast in a bioremediation programme does not have a perception stigma attached as, in laymans terms, we are using brewers yeast, which has been publicly acceptable for decades. Brewers yeast has also been classed as a GRAS (generally regarded as safe) organism. The application of *S. cerevisiae* in bioremediation programmes is, therefore, possible.

The applicability of this research does not relate solely to the environmental sector. Zinc is a structural component of many intracellular enzymes including alcohol dehydrogenase. This enzyme operates in the terminal step of the yeast glycolytic pathway, therefore by manipulating this yeast in order to produce more active ADH, could this improve the overall ethanol yields? If so, this would be very advantageous to the fermentation industry from an economical viewpoint. The use of zinc "loaded" yeast cells, in the form of "Servomyces" products, is currently being investigated as use as a nutritional supplement in the fermentation industry (www.lallemand.com). In addition, during industrial fermentations *S.*

cerevisiae cells suffer various stresses and the introduction of an ion such as zinc (with its many favourable properties) may be able to alleviate some of the stresses. Zinc supplied in the form of "Servomyces" could possibly offer a cheap and favourable solution.

1.10 Techniques available for the detection of zinc

Traditionally, the analytical techniques that exist in order to determine the presence of zinc within the cell were radioactive tracers, namely Zn^{65}, atomic absorption spectroscopy and a chemical method which uses dithizone (Bilinski and Miller, 1983). Novel methods for the determination of metal ions within the cell are becoming available in the form of fluorescence probes.

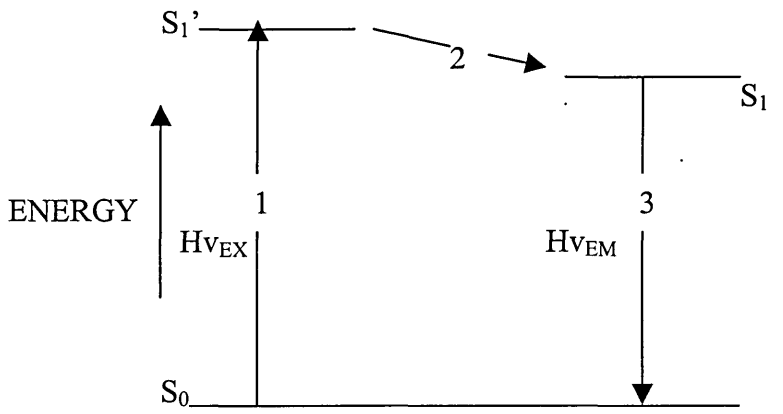
1.10.1 Atomic Absorption Spectrophotometry

Atomic absorption spectrophotometry (AAS) is a technique that was developed in Australia and New Zealand in the 1950's for the analysis of trace metal ions in beer (Frey *et.al.*, 1966). During AAS, the element is not appreciably excited in the flame, but is dissociated from its restrictive chemical bonds, therefore placing the atoms in an unexcited or ground state. The atom, in this low energy level is capable of absorbing radiation at a very narrow bandwidth. A hollow cathode lamp, which is made of the material to be analysed, is used to produce light of a specific wavelength. The light produced from the cathode lamp enters an air-acetylene flame, some of the light is absorbed by the ground state atoms, and become subsequently excited. This absorption results in a net decrease in the intensity of the beam and it is this process which is referred to as atomic absorption (Jensen and Jorgensen, 1996). Since the cathode lamp operates at a discrete wavelength and only detects elements made of the same material as the lamp, interference from other metal ions is limited.

1.10.2 The fluorescence process

The fluorescent process occurs in certain molecules, which have been classified as either fluorophores or fluorescent dyes. The fluorescence principle is best described by the Jablonski diagram. Figure 1.6 is a schematic representation of the fluorescence process, with the individual events depicted.

Figure 1.6: The Jablonski diagram (labels 1: Excitation of electrons, 2: Excited state lifetime, 3: Fluorescence emission)



(Adapted from Johnson, 1996).

The Jablonski diagram demonstrates that fluorescence is a 3-stage process, in which the fluorescent dye is designed to localise within a specific region of a biological specimen or to respond to a specific stimulus (Johnson, 1996).

The first stage begins with the excitation of ground state electrons, this excitation process "warms-up" the electrons, so that they change from the resting ground state (S_0) to a more excited state (S_1'). The electrons move more freely when excited. The excitation process begins due to the supply of an external energy source, *e.g.* lamp. The excited state is, therefore, created due to a chemical reaction. The second phase in the process, which ultimately results in fluorescence emissions, is the excited state lifetime. As the excited state last for a very short period of time the fluorophore undergoes a conformational change from S_1' to S_1. The final, third, stage is the fluorescence emission (hv_{EM}). A photon of energy is emitted and the fluorophore returns to the original ground state (S_0).

Fluorescent probes, or fluorophores, can detect particular components of the cell, with enhanced sensitivity and selectivity than traditional methods. These fluorescent dyes can be separated into 2 forms, permeable and impermeable.

1.11 Novel visualisation techniques for the detection of zinc

1.11.1 Newport Green diacetate

The fluorescent probe Newport Green is a permeable dye, which detects the presence of zinc within the cell. This dye is not exclusive to yeast based studies and other cells types can also be stained using this technique and particular success has been achieved using rat C6 glioma cells (Haase and Beyersmann, 1999), bacterial biofilms (Wuertz *et.al.*, 2000) and neurons (Canzoniero *et. al.*, 1997). Newport Green diacetate distinguishes between bound and free zinc within a cell. This dye favours the free zinc ions (Sensi *et. al.* 1997), thereby allowing for a visualisation technique on the amount of unbound zinc within *S. cerevisiae*. It is, therefore, theoretically possible to determine the locality of free zinc ions within the cells.

There are also fluorescent probes available for the identification of other metal ions *e.g.* Aluminium, Cadmium, Calcium, Cobalt, Iron, Lead, Magnesium, Mercury and Nickel.

1.12 Aims and objectives of this research

S. cerevisiae is known to accumulate zinc and distribute the ions throughout the cell for immediate use, or this yeast may store the zinc in the vacuole until required. This compartmentation and the storage of zinc ions in metallothioneins compounds allows the cells to have a constant supply of zinc when necessary. More recently, through molecular biological techniques, the genes which encode for zinc transport have been elucidated and a more thorough understanding of zinc uptake and transport within the yeast cell has been revealed. However, little research has focussed upon what effect that this accumulation has on cellular physiology. The aim of this research was to investigate the effect that zinc has on certain aspects of yeast cell physiology and also the effect of yeast cell physiology on zinc accumulation, using industrial relevant strains of *Saccharomyces cerevisiae* as the model.

The following chapters aim to determine:

Chapter 3: The uptake and the cellular localisation of zinc by an industrial and a commercial strain of *S. cerevisiae*

Specifically,

- to examine the ability of a zinc specific fluorescent dye to determine location of free zinc within the yeast cell
- To determine the locality of zinc within pre-prepared commercial samples of "Servomyces"

Chapter 4: The influence of physical and chemical parameters on the zinc uptake ability of *S. cerevisiae*

Specifically,

- To determine the effect of physical and chemical parameters on zinc accumulation by industrial strains of *S. cerevisiae*

Chapter 5: Relationship between yeast cell physiological "state" and zinc accumulation

Specifically,

- To examine a strain of lager brewing yeast with respect to zinc accumulation and determine if zinc uptake is growth stage specific.
- To examine how the metabolic "state" of the cell can influence the zinc accumulating ability of a lager strain
- To determine if zinc can alleviate the effects of a chemical and a physical stress on lager yeast

Chapter 6: Relationship between yeast metabolism and zinc accumulation

Specifically,

- To examine the influence of zinc on the production of a specific enzyme, alcohol dehydrogenase,
- To determine the effect of metabolisable energy source and zinc concentration on the production of ethanol.

Chapter 2

General Experimental Methods

2.1 Yeast culture maintenance

The yeast strains that were used throughout this project were industrially relevant strains of *Saccharomyces cerevisiae.* These strains were obtained from the University of Abertay Dundee, Microbial Physiology Research Group, Yeast Division, private collection. The individual strains of *S. cerevisiae* were a lager brewing strain (this strain was a general lager brewing strain of *S. cerevisiae* originally obtained from Dr. B. Taidi, Scottish Courage Brewing Ltd., Edinburgh, U.K.), a distillers strain (DCL "M" yeast, originally obtained from Dr. I. Maynard, Quest international, Menstrie, U.K.), a wine making strain (L-2226, originally obtained from Mr J. McLaren, Lallemand Ltd., Montreal, Canada) and a bakers strain (GB4918, originally obtained from Dr. I. Maynard, Quest international, Menstrie, U.K.). The commercial "Servomyces" product, the cell wall material and the cell fractions were obtained from Dr. J.D. Measham, Institute Rossell, Lallemand Ltd., Montreal, Canada. Pure cultures of these yeasts were obtained using the microbiological streak plate method of single colony isolation onto malt extract agar. The resultant colonies were aseptically transferred onto malt extract agar slopes, which were incubated at 25 ± 2°C for 48 hours, prior to storage at 4°C until required. These slopes were always produced in quadruplicate to give 2 working and 2 reserve slopes.

2.2 Deionisation Procedure

Any glassware utilised in studies concerning accurate determination of zinc concentrations was deionised prior to use. This was necessary in order to remove any contaminant ions. The glassware was soaked in 2% nitric acid for a period of 24 h. then thoroughly rinsed in

deionised water, prior to soaking for 1 h. in 0.1M EDTA. Finally, the glassware was rinsed 3 times in deionised water, to complete the procedure.

2.3 Media design and preparation

The growth media used throughout this research was a hybrid version of Edinburgh Minimal Media (EMM3) (Mitchison, 1970) and a defined yeast propagation media as employed by an industrial yeast manufacturer (information supplied in confidence by Dr. I. Maynard, Quest International, Menstrie, U.K). This hybrid medium, was originally designed by Wardrop (1999) and comprised inorganic salts, sulphate and nitrogen sources, metabolisable energy source, vitamins and trace elements. The zinc concentration of this media was adjusted, dependent upon the particular experiment, in the range 0-102.4μg/ml, by the addition a 14mM stock solution of zinc sulphate heptahydrate (ACS Reagent, which contained no zinc impurities, Sigma chemicals).

No industrial based media (*e.g.* Brewers wort, grape must) were included in this study due to the variation in the zinc and sugar contents, therefore, a chemically defined medium was employed which allowed the zinc concentration to be controlled and adjusted as required.

The concise make up of this growth media is described in Table 4.

Table 4: Yeast propagation defined media (YPDM)

Carbon Source	Either glucose, fructose, maltose or sucrose	30g/l
Nitrogen and sulphur source	Ammonium Sulphate	5g/l
Phosphate source	Ammonium dihydrogen Phosphate	2.84g/l
Potassium source	Potassium chloride	2g/l
Magnesium source	Magnesium sulphate heptahydrate	1g/l
Calcium source	Calcium chloride dihydrate	30mg/l
Zinc source	Zinc sulphate heptahydrate	Variable (0-102.4μg/ml)
Trace Elements	Potassium iodide	0.15mg/l
	Manganese sulphate	0.6mg/l
	Copper sulphate	60μ g/l
	Citric acid	1.5mg/l
	Molybdic acid	0.24mg/l
	Ferric chloride	0.3mg/l
	Boric acid	0.75mg/l
Vitamins	Nicotinic acid	40mg/l
	Inositol	40mg/l
	Calcium pantothenate	4mg/l
	Thiamine-HCl	1.6mg/l
	Pyridoxine-HCl	1.6mg/l
	Biotin	40μ g/l

(All chemicals included in Table 4 were analytical grade, with the exception of the zinc sulphate, which was ACS grade, Sigma Chemicals)

The inorganic components in this formulation were added individually to 500ml of deionised water (16.7 megohms-cm, Barnstead Nanopure II water purification system) with the carbon sources added separately to a further 495ml of deionised water. These components were sterilised by autoclaving at 121° C for 15 minutes at 15psi. This separation of inorganic and organic nutrients in media for autoclaving was necessary to avoid a condensation reaction occurring between the glucose and the amine groups called the Maillard Reaction. The Maillard reaction results in a browning of the media during the elevated temperatures of the sterilisation process. The trace elements were prepared as a 1000-fold stock solution and the vitamins were prepared in a 250-fold concentration stock solution. The trace elements and the vitamins were filter sterilised (Millipore, 0.45μm) and added to the carbon sources aseptically along with the appropriate level of zinc. The inorganic components and the organic were aseptically combined for utilisation in experiments.

2.4 Inoculum preparation

For all experiments the initial culture inocula were of similar size ($5x10^6$ cells/ml; total cell count). This was achieved by inoculating a flask of YPDM with a loopful of the desired organism. The pre-culture was then incubated for 24 hours at a temperature of 25° C ± 2° C on a shaking platform. This facilitated rapid growth of the yeast. Once the seed flask had sufficiently grown an initial cell count and culture viability was established (refer to page 39). The required volume of yeast was aseptically removed from the seed flask and transferred into sterile centrifuge tubes and spun for 5 minutes at 4000g. The supernatant was discarded, and cell pellets washed x3 with deionised H_2O. This facilitated the removal of extraneous zinc ions, zinc loosely bound to the yeast cell walls, the yeast pellet interstitial

space and zinc from the tube itself. Washed cell pellets were then resuspended in 1ml media prior to inoculating experimental cultures.

2.5 Experimental procedure for sampling and acid hydrolysis

Minimal media with various levels of zinc (0, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 102.4 μg/ml) were inoculated at a level of $5x10^6$ cells/ml. The cultures were sampled in duplicate at the following time intervals (0, 6, 12, 18, 24, 30, 60, 180, 360, 1440 minutes). Samples (5ml) were aseptically taken from flasks and centrifuged for 5 min. at 4000g. The resultant pellet was washed 3 times as described above prior to digesting in concentrated nitric acid (1ml, 70%, specific gravity 1.42). When fully hydrolysed (usually after a period of 24 h) the sample was reconstituted to the initial sample volume of 5ml. The supernatants and the digested pellets were then kept refrigerated until analysed by atomic absorption spectrophotometry for total cellular zinc concentration.

2.6 Cell fractionation procedures

The following procedure was carried out in collaboration with Dr J.D. Measham at the Biotechnology Research Institute, Montreal, Canada. In order to fractionate the yeast cell an ultracentrifugation procedure was employed. A sample of yeast cells were disintegrated at 4°C and centrifuged at 1,400g, for 30 mins. This procedure resulted in 2 fractions: the cell wall fraction, which was collected, and also the supernatant. The supernatant fraction was subjected to a further centrifugation process at 40,000g, for 45 mins. at 4°C, again this yielded 2 fractions: a mitochondrial and microsomal fraction. The remainder of the supernatant was precipitated using 5% Perchloric acid and centrifuged at 40,000g for 45 mins. at 4°C. This last step allowed the soluble proteins and the nucleic acids to be precipitated out leaving the amino acids, peptides and salts left.

2.7 Zinc accumulation by a commercial preparation of yeast cell hulls ("Fibosel")

A sample of Fibosel, (Lallemand Inc., Montreal, Canada) was investigated in order to determine the Zn-biosorptive capabilities of a commercial preparation of cell walls (or hulls). YPDM was prepared, and to each preparation, which contained various amounts of zinc from 0-102.4μg/ml. Equal amounts of Fibosel (1g) were then added and at frequent time intervals 5ml samples were aseptically removed and centrifuged at 4,000g for 10 min. The supernatants were removed and stored in deionised sample tubes. The Fibosel pellets were washed x3 with dH_2O, then digested using 1ml of 70% nitric acid. Once digestion was complete, the digested fibosel pellet was reconstituted to the original volume of 5ml, then analysed for zinc content by AAS.

2.8 Desorption experiments

The washing of the cell walls of a lager strain of *S. cerevisiae* was conducted in order to determine the degree of binding of the zinc ions to the cell wall. The lager yeast seed culture was grown in YPDM, and a cell number and a viability assessment conducted. The lager yeast was then inoculated into fresh media containing 102.4μg/ml of $ZnSO_4.7H_2O$, at a repitching rate of approximately $5x10^6$ cells/ml. The cell number and viability assessment was conducted once again, a 6x 5ml sample was then aseptically removed from the culture flask and centrifuged at 4,000g for 10 minutes. The supernatant was removed into a clean, deionised sample tube, and retained for AAS analysis. The cell pellets were washed x10 in either: dH_2O, 0.1M EDTA, 0.25 strength Ringers solution, 0.25 strength Ringers solution containing 0.1M EDTA, 0.1M HCl or 10μM Orthrophenanthroline. After each wash the samples were centrifuged at 4,000g and the supernatants were removed, in preparation for the next wash, the supernatants were retained for analysis of their zinc content by AAS.

2.9 Stress Experiments

In order to determine the effect, if any, that zinc had on stressed cells YPDM was prepared and a seed culture inoculated at a cell density of $5x10^6$ cells/ml. The only exception was that in order to determine the effect of ethanol on the cell, the media was supplemented with ethanol, resulting in a final concentration of 15%. For the heat shock experiment, the cells were incubated in a 45°C water bath for 1 hour after the initial inoculation. The cell number and viability assessment was determined at frequent time intervals (0, 6 and 24 hours). This method was adapted from Walker *et. al.* 1996.

2.10 Analytical Methods

2.10.1 Yeast cell number and viability assessments

Cell number determinations taken over time were indicative of the growth rate of the cultures under the different experimental conditions. Yeast growth also reflected if zinc itself was inhibitory to the yeast. Cell number assessment was conducted at regular time intervals microscopically using a new improved Neubauer haemocytometer. The method of counting utilised was that described by the Institute of Brewing (IoB, 1991). The viability of the cells was determined colourimetrically using citrate methylene blue. Methylene blue (Sigma Chemicals) was dissolved in sodium citrate solution (2%w/v) to a final concentration of 0.01% (w/v) (IoB, 1991). Yeast suspensions were mixed (by vortexing) with an equal volume with the dye, and the percentage viability was determined microscopically using a haemocytometer. The cells if viable have the ability to decolourise the stain through mitochondrial dehydrogenase action, therefore viable cells remained clear and dead cells retained the blue colour of the stain.

2.10.2 Determination of cell biomass by optical density

At frequent intervals, 1ml samples were aseptically removed from culture flasks and the optical density was measured at 540nm in a spectrophotometer (Novospec II LKB). If the optical density was greater than 0.5, a dilution of the original culture was performed.

2.10.3 Dry Weight Analysis

A known volume of yeast cells was centrifuged at 4,000g for 10 minutes, in order to obtain a crisp pellet. The final dry weight was then determined by resuspending the yeast cell pellet with dH_2O, and filtering (Whatman Glass fibre filter, 0.45μM) the suspension through a Buchner funnel. This resulted in the yeast sample being retained on a pre-weighed Whatman glass fibre filter paper. The centrifuge tube was further rinsed with dH_2O to remove any remaining cells. The filter paper was dried using a Mettler LJ16 moisture analyser. Once dry the filter was then re-weighed, therefore the biomass of the initial cell volume could be calculated. Dry weight analysis was always performed in, at least, duplicate.

2.10.4 Protein determination

The determination of the total protein concentration was carried out according to the Bradford method of analysis (Reed *et. al.*, 1998). A 1ml yeast cell suspension of pre-determined cell number, viability and dry weight was centrifuged at 10,000g for 5 minutes resulting in a crisp cell pellet. This resultant pellet was washed in dH_2O and centrifuged once again. This procedure was carried out a total of 3 times in order to remove particulate matter not associated with the cell. The resultant cell pellet was resuspended 1ml of 1M NaOH and incubated at 60°C until a clear hydrolysate was formed. A 100μl volume of the

digested cell pellet was then transferred into sterile microfuge tubes and neutralised using 100µl of 1M HCl. The sample which was mixed by vortexing, and left to stand at room temperature for approximately 3 minutes. To assay for total protein a standard calibration was constructed using bovine serum albumin (BSA). This calibration curve consisted of a series of dilutions of the stock concentration of BSA in the concentration of 100, 250, 400,500,1000 and 1500 µg/ml. The protein content of the yeast cells was then determined by pipeting 0.1ml of the clear hydrolysate into 5.0ml of the Coomassie protein (Pierce Ltd.) assay reagent, and the absorbance was read at 595nm (LKBII, Novospec), using 0.1ml dH_2O into 5.0 ml Coomassie protein assay reagent, as the reference sample. The assay was performed in duplicate in order to gain a mean and a standard deviation. The total protein concentration of the yeast cell was then determined using the calibration curve.

2.11 Ethanol determination

The determination of ethanol in filtered supernatants was conducted using flame ionisation gas chromatography (Hewlett Packard 5710A). The separation was carried out in a 2 metre steel column selective for organic compounds, packed with porapak Q (mesh 80-100), at a temperature of 120°C. Nitrogen was supplied as the carrier gas. Detection of the ethanol was carried out by a flame ionising detector, which was set at 200°C and linked to a Hewlett Packard Series 2 integrator 3996a. The system was calibrated using a 1 % ethanol standard with 5% propan-2-ol, as the internal standard.

2.12 Metal ion determination

2.12.1 Fluorescence dye analysis

Newport Green fluorescent dye (1µg) (Molecular Probes Inc, Oregon, USA.) was dissolved into 50 µl of dimethylsulphoxide (DMSO) to which 50µl of methanol, and 25µl of 2M potassium hydroxide was added. This solution was then mixed by vortexing and the pH

adjusted to 7 with 0.1M hydrochloric acid. This gave a final stock concentration of 10mM. To the yeast cells pellets 200µl of 5µM solution of the fluorescent dye was added. The cells were then incubated at 25°C for 1 hour to allow for maximal loading of the fluorescent dye. Once this incubation time was completed the cells were washed 3x in phosphate buffered saline (PBS), and observed microscopically (x100 magnification) using a fluorescence filter. The image once observed on the microscope was pictured and transferred via a Sony 3CCD camera, onto a computer (Quantimet). The observation and analysis of the image was possible using Q600 (software package).

2.12.2 Atomic absorption spectroscopy

The concentration of zinc ions in the intra- and extracellular samples was determined using a Perkin-Elmer Atomic Absorption Spectrophotometer (AAS) E100. Any dilutions necessary were prepared using deionised H_2O. The cation zinc was determined by AAS using direct aspiration into an air-acetylene flame. The spectrophotometer was calibrated using 4 standard concentrations of the metal zinc (0, 2.5, 0.5, 0.75 and 1.0 µg/ml) and referenced using deionised H_2O.

The set up for the AAS when determining zinc was as follows:

Fuel	: Acetylene (C_2H_2)
Fuel Flow rate	: 2.5ml/min
Oxidant	: Air
Oxidant Flow Rate	: 8.0L/min
Lamp	: Zn
Current	: 15mA
Wavelength	: 213.8nm
Slit	: 0.7nm
Background Correction	: On
Calibration	: Linear AA-BG

The AAS was set-up to automatically provide a mean and a standard deviation of triplicate analysis.

2.13 Alcohol dehydrogenase assay

2.13.1 Preparation of samples

To determine alcohol dehydrogenase (ADH) activity of *S. cerevisiae* a 5mL sample, which was of pre-determined cell number, viability and total protein concentration, was centrifuged at 4,000g for 10 minutes. The supernatant was discarded and cells washed 3 times in dH_2O. In order to prepare yeast samples for the analysis of ADH activity, 1mL of yeast homogenisation buffer was added to the cell pellet. This buffer consisted of 75mM potassium phosphate, 5mM magnesium sulphate heptahydrate, 1mM thiamine pyrophosphate chloride and 1mM 2-mercaptoethanol. The yeast cell pellet, resuspended in buffer was transferred to a sterile microfuge tube, which contained 0.5g. of acid washed glass beads (Sigma, 425-600 microns). The sample was subjected to a series of vortexing for 30 seconds and a 30 second stationary period in which the cells were rested in ice. Once this cycle had been repeated 10 times, the sample was centrifuged at 14,000g for 10 minutes. This homogenate was collected and used to determine the ADH activity of the yeast cells, which were grown under different zinc concentrations.

2.13.2 Assay for the determination of alcohol dehydrogenase within homogenate.

In order to determine the activity of ADH within the yeast cell, the following solutions were added to a clean cuvette: 1.0ml phosphate buffer (0.075M tetrasodium pyrophosphate and 0.05M sodium dihyrogen orthophosphate), 0.3ml 0.01M β-nicotinamide adenine dinucleotide (NAD), 1.0ml 0.03M ethanol and 0.6mL dH_2O. The solutions were mixed by gentle inversion and placed in a 25°C water bath for 2 minutes. The cuvette was dried and

inserted into the spectrophotometer, as this was the reference sample. In order to begin the assay 0.1ml of the homogenised yeast suspension was added. The solutions were quickly mixed by gentle inversion and the absorbance was read at 340nm at 10 second intervals for 3 minutes. This assay was conducted in duplicate in order to determine a mean and a standard deviation of the individual data points.

2.14 Statistical Analysis

All experiments were carried out in duplicate and the analyses were conducted in either duplicate or triplicate dependent upon the experimental conditions. This replication of experiments allowed for a mean and a standard deviation of the individual data points to be calculated and showed that the data was reproducible. Student t-tests were conducted on samples in order to determine whether the results were significantly different. These tests were performed at the 5% level of significance ($p<0.05$).

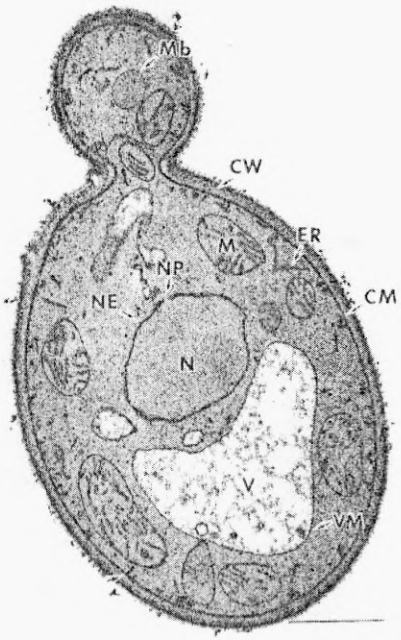
Chapter 3

The uptake and the cellular localisation of zinc by an industrial and a commercial strain of *S. cerevisiae*

3.1 Introduction

The intracellular organisation of a yeast cell allows for a highly dynamic and integrated system. The individual organelles have been intensively studied and the resultant information at the biochemical and genetic levels astounding. The ultrastructure of an "idealised" budding yeast cell is depicted below

Figure 3.1.1: The idealised yeast cell (CW, cell wall; CM, cell membrane; ER, endoplasmic reticulum; VM, valcuolar membrane; V, vacuole; N, nucleus; NE, nuclear envelope; NP, nuclear pore; M, mitochondria)



1μM

(adapted from Osumi, 1998)

The yeast cell wall (Figure 1.4, Chapter 1), has a very important role to play in the biosorption of metal ions. The cell wall of *S. cerevisiae* is a layered structure. This multi-layer component consists of a transparent and amorphous inner layer surrounded by a fibrillar outer layer (Klis, 1994). The inner layer of the cell wall, which is comprised mainly of glucans, is ultimately responsible for providing mechanical strength. The external protein layer, in particular the N-linked mannoproteins, supports the cell wall with regards to its permeability. Although the structure of the cell wall is associated with the terms strength and rigidity, it is a dynamic structure in terms of porosity and elasticity which can adapt to the changing environmental conditions and the growth phase of the cell (Klis, 1996 Remacle, 1990 and Scherrer *et. al.*, 1974).

The binding of heavy metals to the cell wall is possible due to the availability of specific binding regions. These regions may change between species, or they can even change if the yeast is grown in different media (Engl and Kunz, 1995). A brewing strain of *S. cerevisiae* has been shown to concentrate zinc and magnesium ions in the mannoprotein section of the cell wall (Mochaba *et. al.*, 1996). The sulphydryl groups of cysteine, which are located in this protein fraction, have been implicated in the binding of the individual zinc ions (Brady and Duncan, 1994; Mochaba *et. al.*, 1996).

Specific organelles within the cell, which are known to remove zinc ions from the cytoplasmic pool, are the mitochondria and the nucleus. The removal of ions from the cytoplasm is due to the necessity of these ions within these specific organelles and to maintain cellular homeostasis of Zn. Within the nucleus zinc plays a role in stabilisation of nucleic acids and the ions are incorporated into DNA and RNA polymerase enzymes (Faillia, 1977). A single zinc ion is also incorporated into a cysteine rich amino acid loop, which intercalates directly into the DNA helix. This structure is classed as a zinc finger.

The mitochondrion, is the site of oxidative metabolism within the yeast cell, zinc ions which are transported into this membrane bound organelle may be utilised in many of the enzymes present *e.g,* pyruvate dehydrogenase (Voet and Voet, 1990).

3.1.2 Servomyces Zinc

"Servomyces zinc" is a commercially prepared strain of *S. cerevisiae* that has been grown in the presence of excess organic zinc salts. Within this preparation the yeast has sequestered high levels of the available Zn ions. The Rossell institute of Lallemand (Montreal, Canada) is producing the Servomyces zinc product as a potential nutritional supplement for industrial fermentations and as a human dietary supplement (along with other mineral-enriched yeasts, such as Servomyces- Mg, Ca, Mo and Se (Dr F.R. Wardrop, personal communication). The advertised benefits in brewing fermentations of this product (announced by Whitelabs website, www.whitelabs.com) include improved fermentation rates and ethanol yields, reduction in undesirable aftertastes. It is also reported to improve the health and the viability of the yeasts employed in brewing. This product is added to the fermentation at the mashing stage, where the "Servomyces" products are used in a sacrificial role and boiled to release their nutrients to the surrounding environment. These products aim to supplement an industrial media that may contain sub-optimal concentrations of essential nutrients.

3.1.3 Experimental Aims

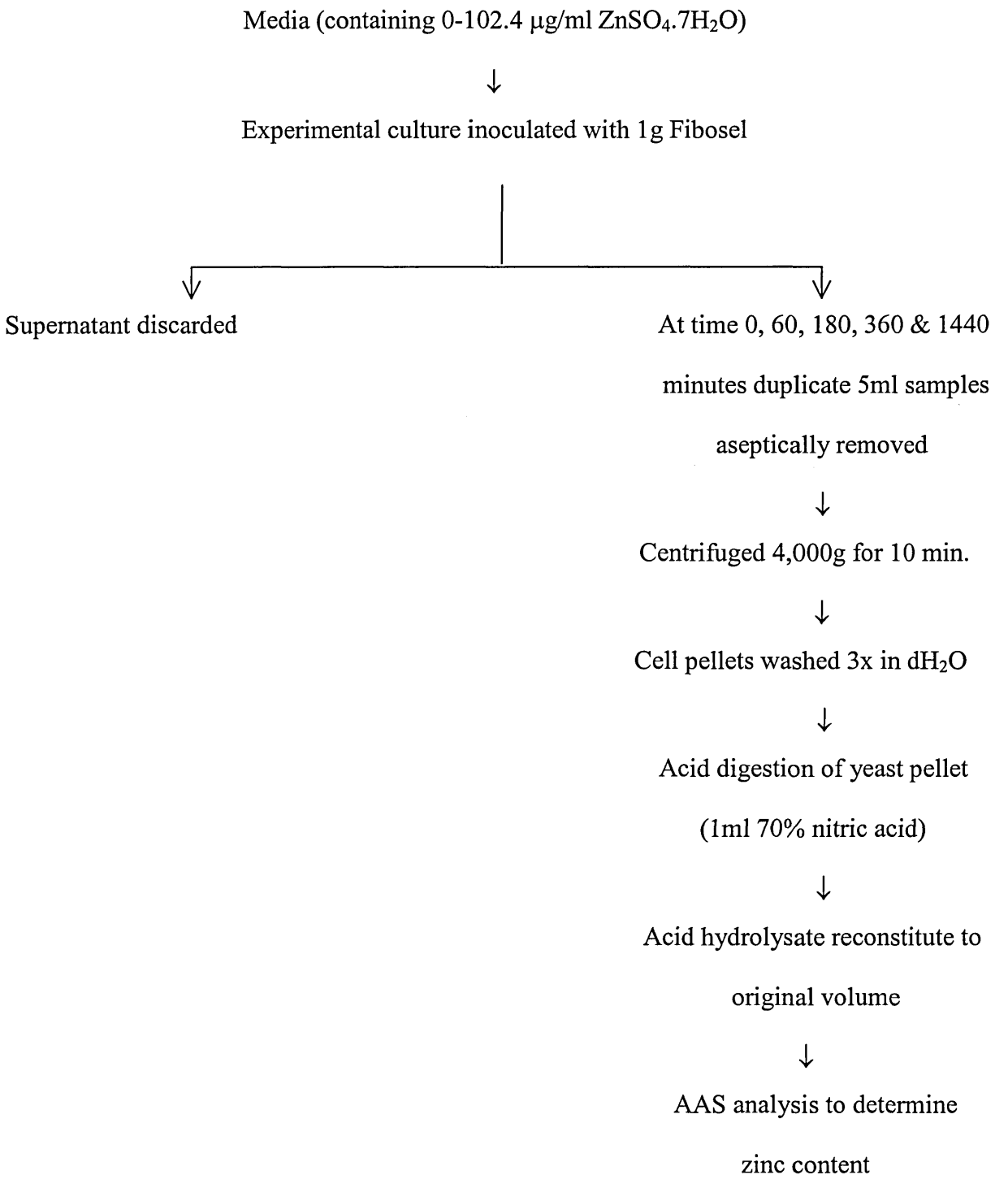
The aim of this chapter was to study a commercial preparation of zinc enriched yeast products, with respect to determining the cellular location of zinc ions, in whole cells and in fractionated preparations. The use of a fluorescent dye, Newport Green, was investigated in order to determine if it could detect the locality of the zinc ions within the cell without going to labour intensive and expensive fractionation processes.

3.2 Experimental Approach

The yeast culture that was employed throughout this Chapter were the commercial Servomyces zinc product (0305C and 0055C) a lager brewing strain of *S. cerevisiae* supplied by Scottish Courage Ltd. The lager yeast was grown in YPDM in seed cultures and then pitched into fresh media at approximately $5x10^6$ cells/ml. The Servomyces products and "Fibosel" (a commercial preparation of cell hulls, obtained from Dr. J.D. Measham, Rossell Institute, Montreal, Canada) were inoculated into dH_2O, prior to analysis of intracellular zinc level, cell number, viability assessment and dry weight.

3.2.1 Zinc accumulation by a commercial preparation of cell walls (Fibosel)

The following flow diagram describes the approach to determining the zinc accumulating ability of Fibosel.



3.2.2 Desorption of zinc from the cell walls of an industrial strain of *S. cerevisiae*

The following diagram summaries the experimental approach adopted to determine the degree of binding of the zinc ions to the cell wall of lager brewing yeast.

Seed culture of lager brewing yeast

↓

Cell number and viability assessed

↓

Culture inoculated into minimal media containing a known amount of $ZnSO_4.7H_2O$

↓

Cell number and viability assessed

↓

Samples (5mL) removed

↓

Centrifuged at 4,000g, for 10 minutes

↓

Samples washed x10 in either: dH_2O, 0.1M EDTA, 0.25 strength Ringers solution, 0.25 strength Ringers solution containing 0.1M EDTA, 0.1M HCL or 0.1M Orthrophenanthroline

↓

Supernatants retained

↓

Supernatants analysed for zinc content by AAS

3.2.3 Cell Fractionation Procedure (in collaboration with Dr. J. Measham, Institute Rossell, Canada)

The following diagram summaries the yeast cell fractionation procedure of the commercial "Servomyces" zinc product.

Commercial preparation of Servomyces zinc product

↓

Disintegrated at 4°C and centrifugation at 1400g for 30 mins.

↓

Supernatants decanted and cell wall fractions retained on ice

↓

Supernatants centrifuged at 40,000g, 4°C for 45 mins.

↓

Supernatant decanted and mitochondria

and microsomes fractions retained on ice

↓

5% Perchloric acid added to supernatant

↓

Centrifugation at 40,000g, 4°C for 45 mins.

↓

Contained in the precipitate were soluble proteins, nucleic acids, amino acids,

peptides and salts were contained in solution

3.2.4 Fluorescence staining of a commercial and an industrial strain of *S. cerevisiae*

Yeast seed culture

↓

Cell number and viability assessed

↓

Culture inoculated

↓

Cell number, viability, protein and dry weight analysed

↓

Samples (1mL) taken

↓

Centrifuged at 10,000g, for 5 minutes

↓

Cells incubated in 200μL of 5μM newport green diacetate
for 1hour at 25°C

↓

Centrifuged at 10,000g for 5 minutes

↓

Washed x3 in 0.1M phoshpate buffered saline

↓

Reconstituted cells in known volume of PBS

↓

Visualised cells using a Leica microscope
containing a fluorescent filter

↓

Captured images on a Sony 3CCD camera

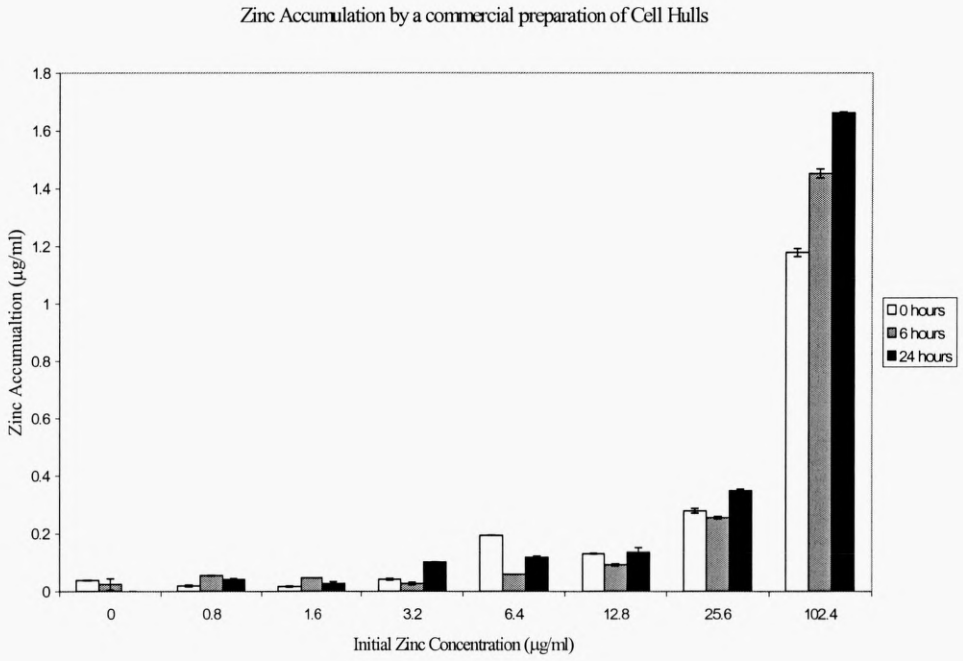
↓

Analysed images using Quantimet Q600 software

3.3 Results and Discussion

The uptake of zinc ions by *S. cerevisiae* is known to be a biphasic process consisting of a metabolic independent stage and a metabolic dependent stage. The ability of the cell wall to sequester ions is well documented (de Rome, 1988, Remacle, 1990, Brady and Duncan, 1994, Volesky and May-Phillips, 1995). However, is the ability of isolated cell walls to remove ions just as successful? Figure 3.3.1 demonstrates the ability of a commercial preparation of cell walls to remove zinc from an aqueous solution.

Figure3.3.1: Determination of a commercial preparation of *S. cerevisiae* cell walls (Fibosel) to accumulate zinc over time from YPDM containing varying initial concentrations of zinc (0-102.4 μg zinc/cell)



The results demonstrated increasing removal over time, in the higher zinc concentrations, with the maximum zinc removed from the solution approximately 1.7 μg/ml. This was considerably lower than when the cells were whole and viable. However, it is possible that the cell walls were damaged during the removal process, resulting in a reduction of the available binding sites. Later results demonstrated (chapter 4) that maximal uptake was achieved when the cells were viable. Dead biomass and cell wall preparations were not able to actively accumulate metal ions. Therefore, without manipulation metal accumulation by cell wall biomass alone was always lower.

Upon statistical analysis of the results using a t-test, the 24 hour data concerning zinc uptake from initial zinc concentrations of 25.6 μg/ml and 102.4 μg/ml demonstrate that the uptake is significantly different ($p<0.05$), therefore it can be stated that there is increased zinc removal by the cell hulls at 24 hours.

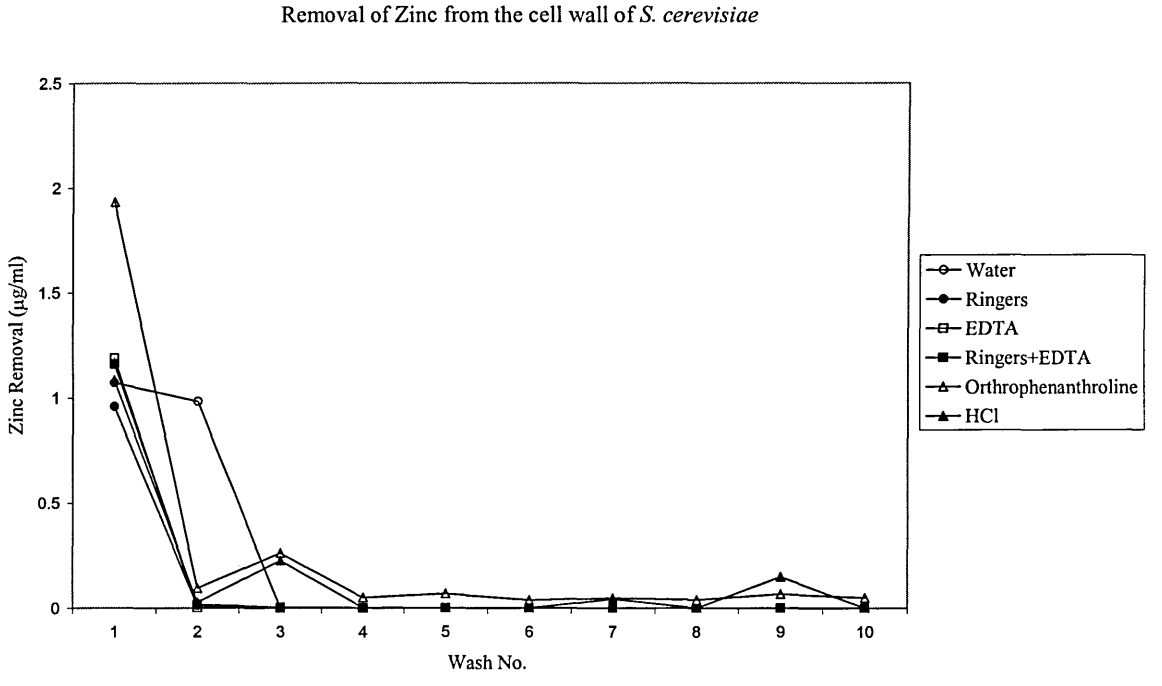
This experiment was conducted using Fibosel- a commercial preparation of cell walls. Therefore, the prospect of using the cell "hulls" in a bioremediation process could be re-examined. For example, in situations where the waste effluent contained very low concentrations of contaminants *i.e.* zinc ions.

The uptake of metal ions onto the cell wall of *S. cerevisiae* was rapid and occurred almost instantaneously. However, what was the degree of this binding? Researchers studying the biosorptive effect of microbial biomass have stated that metabolism independent ion accumulation is both rapid and reversible (for review see: Gadd, 1993).

Figure 3.3.2 demonstrated the effect of different wash buffers on the ability of a strain of lager yeast to release metal ions from the cell wall. The buffers used were deionised H_2O, 0.25 strength Ringers solution containing 0.1M E.D.T.A., 0.1M HCl, as well as general and

specific chealating agents (0.1 M EDTA and 10μM orthrophenantroline, respectively). The latter is a strong and specific chelator of zinc ions (Walker and Duffus, 1979).

Figure 3.3.2: Graphical representation of the desorption of zinc ions from the cell wall of *S. cerevisiae* at zero time, from an initial zinc level in the media of 102.4 μg/ml, and the uptake was 408fg zinc/ cell, using various wash buffers.



Since the primary site of metal ion uptake is the yeast cell wall, this experiment attempted to determine the degree of surface binding. The samples for this experiment were taken at zero time, and, Figure 3.3.2 shows that the binding of zinc ions to the cell wall was both rapid and strong, with very little zinc being removed by the washing agents. The wash

solutions including water removed very little of the total cellular zinc. The most successful washing buffer was orthrophenanthroline (10μM). This chealating agent exhibits high selectivity for zinc, and has been used to remove zinc from zinc containing metalloenzymes (Walker and Duffus, 1979). With all the washing agents the removal of zinc ions levelled off after 2-3 washes. Error bars are not included on the graph for clarity. However, the experiments were repeated (and data presented in Figure 3.3.2 are means of 3 experiments). It was therefore possible to determine that the binding of zinc ions onto the cell was extremely rapid, occurring almost instantaneously, and the poor removal demonstrated that in this situation the removal of zinc ions was not fully reversible.

Muraleedharan *et. al.* (1991) reported the removal of zinc from cell walls of *S. cerevisiae* using 0.1M HCl. However, Figure 3.3.2 shows that the removal of zinc using 0.1M HCL with this strain was similar to the results given for the control (dH_2O). Therefore, the acid as a washing solution was rather unsuccessful. The differences that have been reported may be accounted by subtle differences in the cell walls of the two different *S. cerevisiae* strains, or even the growth stage of the cells. Growth in different media can affect the composition of the yeast cell wall by creating different binding sites (Engl and Kunz, 1995). Therefore, a direct comparison in this instance is of little use.

The ability of whole cells of *S. cerevisiae* to sequester heavy metals is well documented (Akthar *et.al.*, 1996; Fuhrmann and Rothstein, 1968; Gadd 1992, Okorokov *et. al.*,1977; White *et.al.*, 1995) with the compartmentalisation of ions into the vacuole now understood (Nies, 1999; Okorokov *et. al.*, 1985; Ramsay and Gadd, 1997). However, upon analysing the zinc content in the commercial preparations of both the viable and non-viable

"Servomyces" zinc preparations, as well as the cell walls, the mitochondria fractions and the cell hulls, an insight into the locality of the ions within these cells may be established.

The "Servomyces" products were loaded with zinc under different physiological conditions. The products referred to as "Servomyces" viable were viable *S. cerevisiae* cells loaded with zinc, likewise the "Servomyces" non-viable product was a dead preparation of cells loaded with zinc. The terms "Servomyces" viable and non-viable were, therefore, referring to the physiological state of the cell upon zinc loading.

Figure 3.3.3: Comparison of the zinc content from whole and separated cellular fractions from the commercially available Servomyces zinc product.

	Product/ Cell Fraction	Mean Dry Weight (g/L)	Mean Protein Conc. (μg/mL)	Mean Zinc Content (μg/mL)	μg Zinc/ μg Dry Wt./ Protein	Newport Green Staining Picture Ref. No.
Whole Cells	ServoZn Viable (0305C)	1.595	-	887 (0.012)	556.1	Figure 3.3.4
	ServoZn Non-Viable (0055C)	1.35	-	191.95 (0.106)	142.2	Figure 3.3.5
Cell Fractions	Cell Hulls	1.465	-	0.585 (0.006)	0.4	Figure 3.3.6
	Cell Walls (0305C)	1606.5	-	6,480 (0.011)	4.03	-
	Cell Walls (0055C)	94.3	-	10,030 (0.015)	106.4	-
	Mito. (0305C)	-	66.26	41.4 (0.006)	0.62	-
	Mito. (0055C)	-	64.34	54.4 (0.003)	0.85	-

(Standard deviations if included in Figure 3.3.3, are to be located under the mean value, in parenthesis)

The penultimate column in Figure 3.3.3 is the most interesting, as it clearly demonstrated that the viable preparation had a much greater zinc content (556.1 μg zinc/μg dry wt.) than the non-viable Servomyces product (142.2 μg zinc/μg dry wt.). The increase in total zinc content was approximately 390% greater in the viable sample as compared to the non-viable sample. The viable sample had a very small proportion located on either the cell wall or in the mitochondria and microsomal fraction. The zinc was, therefore, located elsewhere within the cell, with the likely-hood that it was in the vacuole. White and Gadd (1987)

demonstrated that of the total cellular zinc, approximately 56% was located in the soluble vacuolar fraction, with 39% bound to insoluble cellular components and 5% located in the cytosolic fraction. The non-viable Servomyces product, however, demonstrated a different pattern of uptake, with the total amount of zinc removed from the growth environment totalling 142.2 μg zinc/μg dry weight. The cell wall was found to contain approximately 75% of the total zinc.

In determining if the zinc content associated with the "Servomyces" samples and whether the zinc content of the prepared fractions were significantly different a student t-test was conducted. The "Servomyces" viable and non-viable whole cell preparations were compared as were the cell wall and the mitochondrial fractions, with the null hypothesis being rejected in each case ($p<0.05$).

The results from these tests concluded that the sample means were statistically different ($P<0.05$). Therefore, in comparing the results from Figure 3.3.3, it is obvious that there was a significantly different pattern of uptake between the two samples. The viable sample accumulated much more zinc and distributed it internally as opposed to retaining the majority of the ions on the cell surface like the non-viable zinc loaded preparation.

The staining of an industrial strain and a commercial "Servomyces zinc" preparation, and "Fibosel", a preparation of cell wall material was carried out in order to determine the free zinc within the preparations. This was achieved by loading the cells up with 5μM Newport Green Diacetate. The cells were loaded with 5μM Newport Green due to the weak fluorescence emission of the fluorophore alone (in comparison to other concentrations). These preparations are all strains of *S. cerevisiae*, and the data that refers to the pictures are shown in Figure 3.3.3.

Figure 3.3.4: "Servomyces" viable product stained using 5μM Newport Green Diacetate, magnification x100.

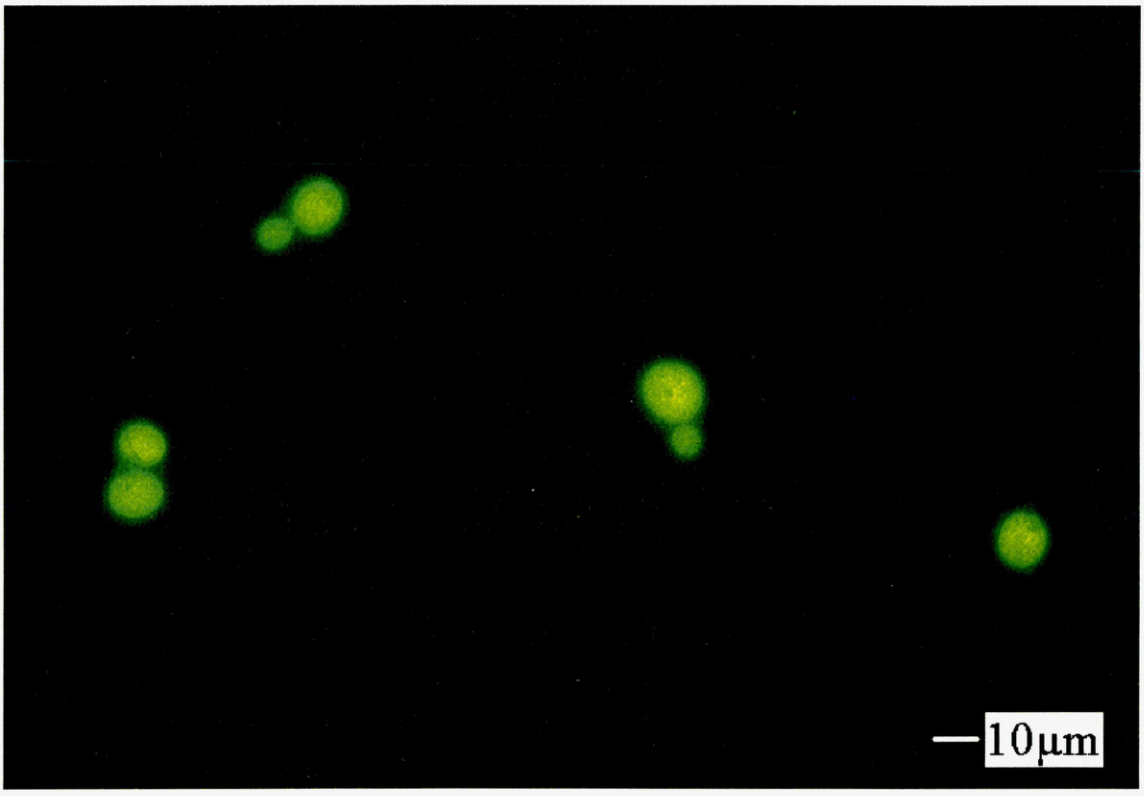


Figure 3.3.5: "Servomyces" non-viable product stained using 5μM Newport Green Diacetate, magnification x100.

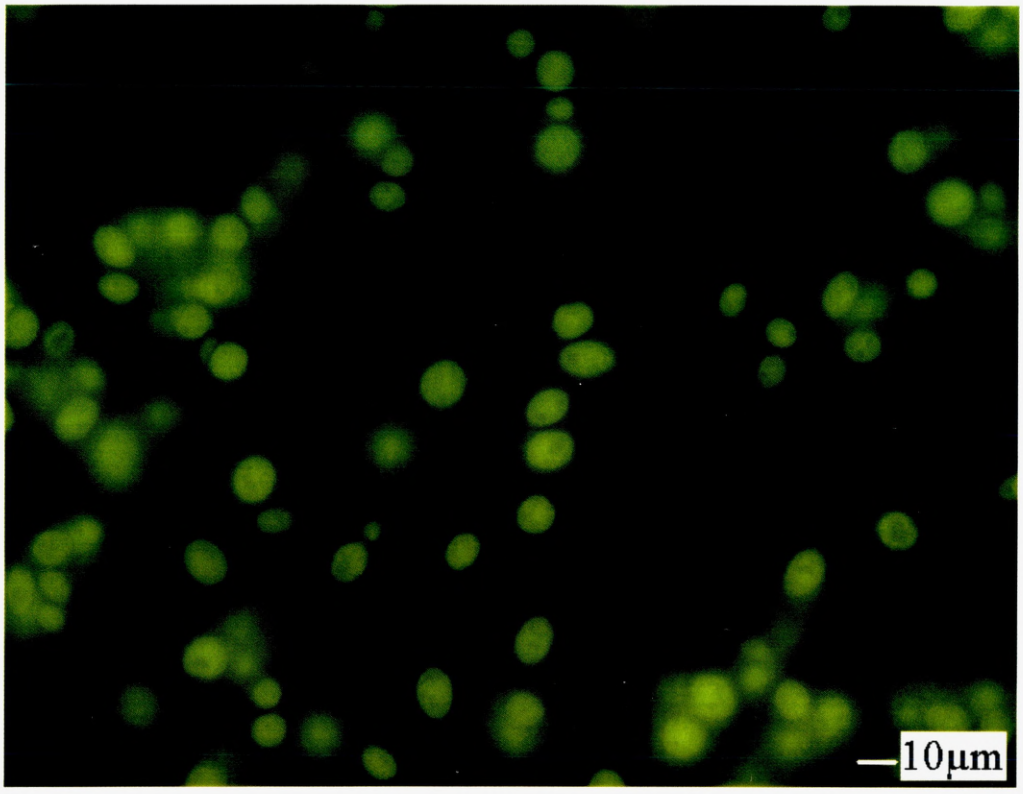


Figure 3.3.6: Cell hulls product, unsupplemented with zinc and stained using 5μM Newport Green Diacetate, magnification x100

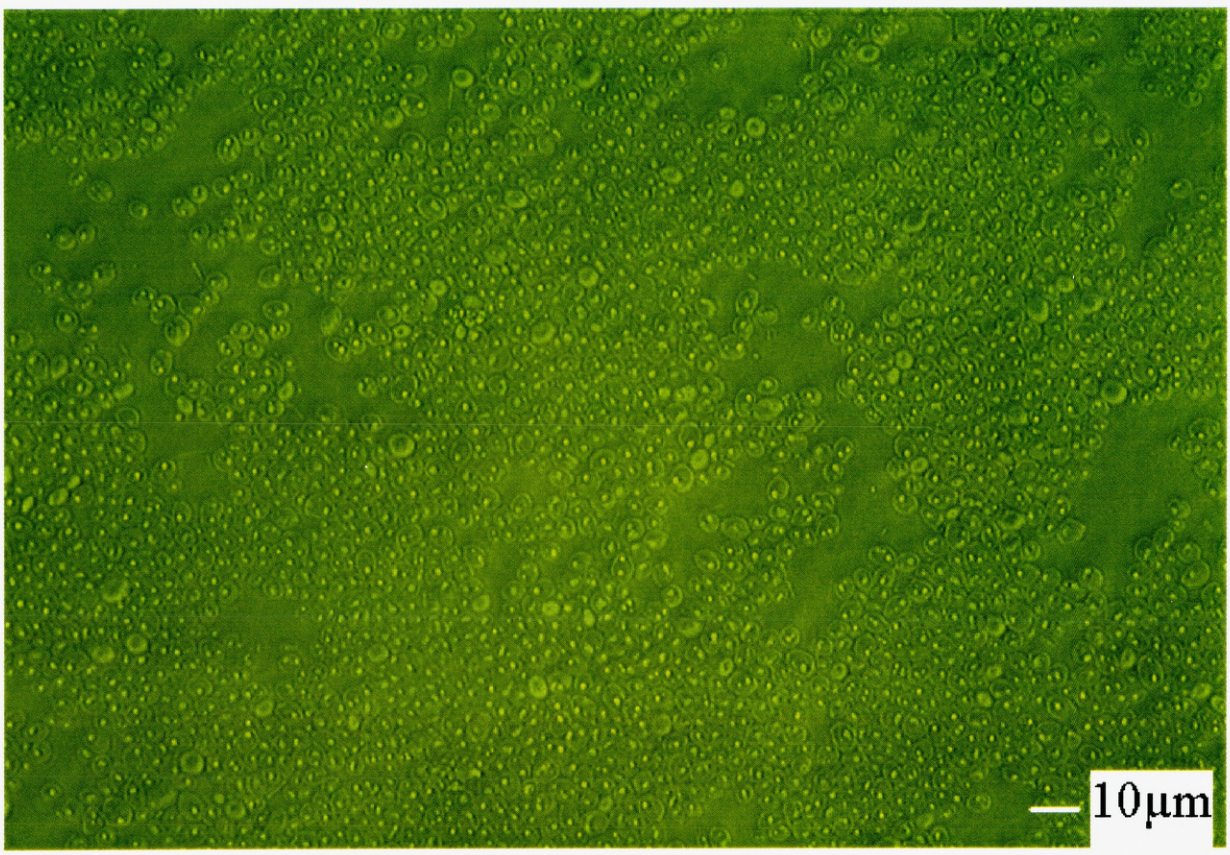
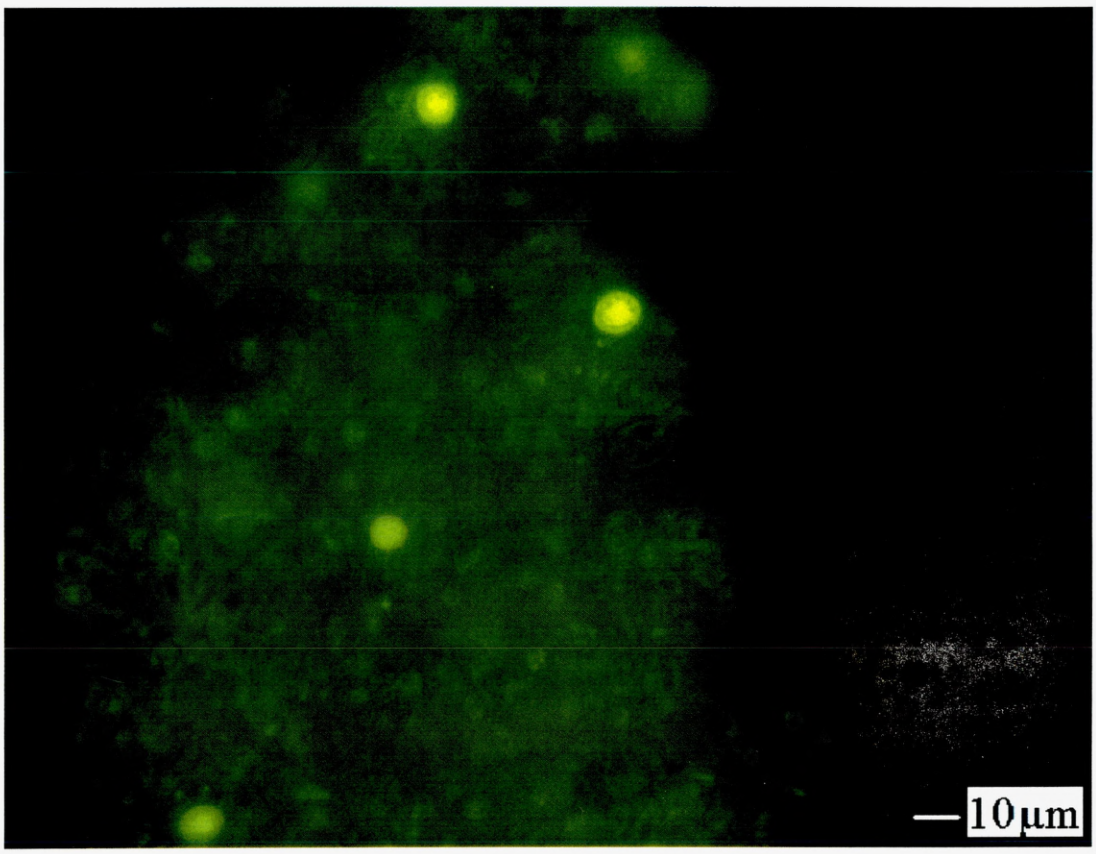


Figure 3.3.7: Cell hulls product supplemented with 1M $ZnSO_4.7H_2O$, stained using 5µM Newport Green Diacetate, magnification x100.



As previously mentioned, due to the biological necessity of zinc, the amount of free zinc in viable cells is maintained relatively constant but extremely low (ranges from 1-5% of free zinc is found within the cell: Simons, 1993; White and Gadd, 1985). Therefore, a rough estimation can be assessed of the final zinc content of the cells depending upon the amount of free zinc within the yeast cell. The fluorescent images (Figures 3.3.4-7) have highlighted the amount of free zinc within the commercial Servomyces zinc product. The data referring to these images is shown in Figure 3.3.3. In order to quantify the amount of free zinc within the calls, the fluorescent image can be transferred into a "mono-image". This "mono-image" will allow the determination of a *grey value* of each pixel within the image of that cell. This results in a mean and a standard deviation calculated on the *grey value* within the population of the cells. The difference in the *grey values* between the viable (2.16) and the non-viable (41.57) Servomyces products, allowed a comparison of the amount of free zinc associated with the cell population. Therefore, although the AAS demonstrated that the viable product contained a much greater amount of total zinc, the *grey determination* has shown that the non-viable sample contains a much greater quantity of free ions. Haase and Beyersmann (1999) reported that a decrease in the viability of rat glioma cells resulted in an increase in the fluorescence within both the cytosol and the nucleus. This increase in the fluorescence between equally loaded zinc containing cells could be due to the release of bound zinc ions from the organelles during apoptosis. The counter-staining of individual organelles with specific stains (*e.g.* the nuclear stain, DAPI) was not possible due to the incompatibility of the two products.

Figure 3.3.8: Quantification of fluorescence from Newport Green diacetate stained cells from whole and separated fraction of the commercial Servomyces preparation.

	Product/ Cell Fraction	Mean Zinc Content (μg/mL)	μg Zinc/ μg Dry Wt./ Protein	Grey Value	Newport Green Staining Picture Ref. No.
Whole Cells	ServoZn Viable (0305C)	887 (.012)	556.1	2.16 (16.81)	Figure 3.3.4
	ServoZn Non-Viable (0055C)	191.95 (0.106)	142.2	41.57 (25.11)	Figure 3.3.5
Cell Fractions	Cell Hulls	0.585 (0.006)	0.4	-	Figure 3.3.6
	Cell Walls (0305C)	6,480 (0.011)	4.03	-	-
	Cell Walls (0055C)	10,030 (0.015)	106.4	-	-
	Mito. (0305C)	41.4 (0.006)	0.62	-	-
	Mito. (0055C)	54.4 (0.003)	0.85	-	-

Unfortunately, the visualisation of the cell wall material (unsupplemented with Zn), Figure 3.3.6, and the mitochondrial fraction (not shown) was not possible due to the weak fluorescence. This may be interpreted as follows: either the zinc within these fractions was all bound (as the AAS result demonstrated that there was a small amount of zinc within these fractions), or that the fluorescence was simply too weak for the transfer of the image from the microscope to the image analyser. Upon comparing the *grey values* (Figure 3.3.8) statistically using a student t-test, it was found that the results were not statistically different ($p<0.05$). This may be interpreted as either all of the zinc was bound up either in the cell wall fraction or intracellularly within proteins, nucleic acids and enzymes or possibly

compartmentalised within the vacuole. Therefore the distribution between viable and non-viable cells loaded with zinc was different, and although results demonstrated differential distribution the amount of free zinc within the samples was not significantly lower. Again determining that zinc ions are tightly bound to the cell wall, or utilised within the cell or compartmentalised until required.

3.4 Conclusion

The main points to conclude from this Chapter are that zinc uptake onto the cell wall was rapid and strong, with very little removed by specific and general chelating agents. Maximal uptake of Zn ions was demonstrated in the commercial "Servomyces" samples when the yeast was loaded when still viable. In this viable sample the zinc was located inside the cell (as a very small proportion was located on the cell wall). However, when the non-viable product was loaded with zinc the majority of the ions were located on the cell wall. This was due to the dislocation of the transport processes that usually aid the transfer of zinc from the cell wall into the cytosol. The use of fluorescent techniques to examine the amount of free zinc within the yeast cells had varying degrees of success. It can be concluded that the amount of free zinc within the cell is extremely low, and the amount of free zinc associated with the different "Servomyces" products was not statistically different. The visualisation of whole cells was possible, however, the ability of either the dye to stain organelles or the imaging equipment to visualise such small particles limited the applicability of the technique in this instance.

In order to determine further the transport of zinc across the plasma membrane in viable cells the use of *ZRT 1* and *2* deficient *S. cerevisiae* mutants may be employed. Through the use of these mutants the genes which control zinc transport across the plasma membrane would be silenced and a different pattern of uptake should be apparent, and possibly visualised via the Newport Green staining.

Chapter 4

The influence of physical and chemical parameters on zinc uptake by *S. cerevisiae*

4.1 Introduction

The physical and chemical environment is very influential in dictating yeast growth and metabolism. The physiology of *S. cerevisiae* is dependent upon the availability of specific nutrients and their translocation into the cell. The processes of metal ion biosorption and bioaccumulation are also influenced by external conditions. Biosorption is effected by temperature and pH fluxes, whereas bioaccumulation is reliant upon the presence of a metabolisable carbon source. The catabolism of the carbon source will produce energy and within the cell carbon sources are major structural elements.

4.1.1 The effect of carbon source on zinc accumulation by *S. cerevisiae*

In this chapter, the carbon sources supplied in the yeast growth medium were, either: glucose, fructose, maltose or sucrose. These substrates were added to stimulate growth and development of the culture. The presence of a metabolisable carbon source within the cell will, amongst other things, power the plasma membrane ATPase, which in turn allows zinc transport into the cytosol by active transport.

4.1.2 The effect of temperature on *S. cerevisiae*

Physical conditions do influence the uptake of ions into the cell, and temperature can also severely affect the growth and viability of *S. cerevisiae*. During low temperatures, *e.g.* 4°C, the microbes enter a period of dormancy, and the cellular water is affected. Water is an essential cellular requirement in all organisms. Its function is to aid the movement of

solutes throughout the cell and allows for enhanced intracellular enzyme activity (Walker, 1998a). Water is transported into the yeast cell by diffusion channels (for diagrammatic representation see: figure 1.1c).

Therefore, during low temperatures the metabolic activity of the cell is inhibited and the rate at which chemical reactions occur is significantly reduced. At this lower temperature the water viscosity within the cells is increased, proteins are normally denatured and the relative permittivity of water is also increased (Smith, 1993). An increase in the water permittivity will effect zinc accumulation, as a consequence of this increase there will be a reduction in the attraction between ions. The greater increase within the cellular water permittivity, means that water ions will become more positively charged, therefore, repelling the positively charged zinc ions.

The plasma membrane also undergoes remarkable changes at this low temperature. The normal disarrayed fluid structure of the plasma membrane changes in favour of a more arranged crystalline formation, which researchers refer to as a sol-gel structure (Farger and Smith, 1995). This change in the structure of the plasma membrane from the disorganised fluid model to the organised sol-gel structure, is attributed to the alteration in the ratio of unsaturated to saturated fatty acids (Berry and Foegeding, 1997). This change is in favour of the unsaturated fatty acids. This alteration in the fatty acid composition of the plasma membrane is called the lipid transition state, and the temperature at which it occurs is called the transition temperature (Berry and Foegeding, 1997). A rapid decrease in temperature has been shown to result in cold shock injury in a brewers strain of *S. cerevisiae* (Fargher and Smith, 1995). Although evidence for heat shock proteins in yeast is frequently reported, the production of cold shock proteins is less well understood. Kaul *et. al.* (1992) reported the existence of a 33kDa protein induced by cold temperature. This protein was detected in a strain of *S. cerevisiae* which was subjected to a temperature of 10°C after a 30

minute period. *S. cerevisiae* may be protected from the detrimental effects of long term storage by supplementing the cells with cryopreservants *e.g.* glycerol. Walker (1998b) has investigated the effect of extracellular magnesium with respect to protecting the cells from cold shock in a lager strain of *S. cerevisiae*. This research demonstrated a positive influence of magnesium ions in cells that were subjected to temperature shock (heat and cold shock).

4.1.3 The effect of pH changes on *S. cerevisiae*

Most yeast strains grow within the pH range 4.5 to 6.5. This range is not specific, as most yeast species are capable of growing in slightly more acidic or basic conditions. The ability of yeast cells to sequester zinc ions is, however, dependent upon the pH of the surrounding environment. Fuhrmann and Rothstein (1968) noted that zinc accumulation by *S. cerevisiae* was dependent upon pH, with maximal sequestration occurring when the growth media was between pH 5-6. Anything below this and uptake was markedly reduced. Studies of the effect of pH on the ability of *C. albicans* to accumulate zinc found that zinc uptake decreased when the pH value was increased (Faillia *et. al.*, 1976; Faillia and Weinberg, 1977). Zinc uptake at pH greater than 6.8 was reduced due to the formation of complexes with polyphosphates, carbonates and hydroxides (Ross, 1994). The pH of the media during these studies was pH4.5, however, the pH of the media throughout the experimental procedures decreased quite significantly. This was probably due to the extrusion of protons and the production of metabolites which are secreted into the media. It can be stated that the pH of the surrounding environment was extremely influential in studies of zinc uptake.

The experimental aims of this chapter were:

Specifically

- To determine the effect of physical and chemical parameters on zinc accumulation by industrial strains of *S. cerevisiae.*

The industrial strains of *S. cerevisiae* examined were: lager brewing, distilling, wine and baking strains (for original sources of the yeasts, see chapter 2).

4.2 Experimental approach

Yeast seed cultures were grown up in YPDM for a period of 24 hours. Cell number and viability assessment were conducted on the seed culture and cells were re-inoculated into fresh YPDM at 25°C ± 2°C, which contained different initial concentrations of $ZnSO_4.7H_2O$ (see Table 4, Chapter 2) at a total cell density of $5x10^6$ cells/ml. At frequent time intervals duplicate 5ml samples were aseptically removed from culture flasks and centrifuged at 4,000g for 10 min. Resultant cell pellets were washed 3x in dH_2O, the supernatants discarded and the pellet digested in 1ml 70% nitric acid. Once digestions completed, hydrolysates were reconstituted to the original sample volume of 5ml, and the zinc content analysed by AAS (full details see, chapter 2). At the same time as the samples were taken in order to determine the total cellular zinc content, the cells were examined with respect to the cell number and viability.

For experiments that studied the effect of temperature on the ability of industrial strains of *S. cerevisiae* to sequester zinc the following adaptations to the above experimental approach were conducted. For temperature studies the cells were inoculated into minimal media that was pre-chilled to 4°C, and the temperature remained was maintained for the remainder of the experiment.

4.3 Results and Discussion

The growth and the viability of 4 industrially relevant strains of *S. cerevisiae* were examined when the minimal media was supplemented with various metabolisable energy sources (glucose, fructose, maltose and sucrose). During the lager yeast growth studies the cultures followed the growth curve through the lag, exponential and stationary phase of growth, and it is also assumed that the other strains also followed a similar pattern of growth. The influence of these energy sources on the ability of these strains to sequester zinc was also examined.

4.3.1 Expression methods for the accumulation of zinc

Zinc accumulation may be expressed as mg dry weight/min (Mowll and Gadd, 1983), nmol zinc/ 10^7 cells (White and Gadd, 1987), ppm (Densky *et. al.*, 1966), mg zinc/ kg^{-1}(Yazgan and Őzcengiz, 1994) or fg/cell (Walker *et. al.*, 1996). Fg zinc/cell is the expression method of choice throughout the results section, which correlates, cell number as an assessment of growth with total cellular zinc. Therefore, allowing an expression method which demonstrated zinc on a per cell basis. If the culture was growing, then after a period of 24 hours in some cases it may appear that the cells were releasing zinc back into the media (in comparison to the results obtained for the 6 hour study). However, in reality there was more zinc accumulated, and an observed decrease is solely due to the mathematics involved in the expression system (for example see Figure 4.3.2). Therefore, throughout the following discussion of results the total amount of zinc sequestered by the population (μg zinc/ml) may be referred to as well as how much zinc that the individual cell accumulated (fg zinc/cell).

Figure 4.3.2: Table demonstrating the difference between the expression methods of fg zinc/cell, μg zinc/ml and total amount of zinc accumulated expressed as a % of the initial zinc concentration from *S. cerevisiae* lager cells which were grown in YPDM containing 3% glucose after a period of 24 hours.

Expression Methods	Initial zinc concentration (μg zinc/ml)							
	0	0.8	1.6	3.2	6.4	12.8	25.6	102.4
Zinc Uptake (fg zinc/cell)	4.7	11.48	15.05	20.73	50.28	78.25	39.26	120.73
Zinc uptake (μg zinc/ml)	0.092	0.267	0.474	0.881	1.521	2.206	2.719	3.803
Uptake as % of the available zinc	0	33.38	29.62	27.53	23.76	17.23	10.6	3.71

4.3.3 The influence of metabolisable energy source on growth and zinc accumulation by industrial strains of *S. cerevisiae*

4.3.4 *S. cerevisiae* lager brewing yeast.

Lager yeast is generally supplied with brewers malt wort as an industrial based carbon source. This growth medium is highly complex and not fully defined (Maskell *et.al.* 2001). The main carbohydrate components of brewers wort; are fermentable sugars (a mixture of glucose, fructose, sucrose, maltose and maltotriose), and the non-fermentable dextrins. The lager yeast grew well in defined media with 4 metabolisable energy sources supplied (Figures 4.3.4.1a, 4.3.4.2a, 4.3.4.3a and 4.3.4.4a: glucose, fructose, maltose and sucrose respectively), when the temperature was 25°C ± 2°C, and the pH was 4.5. These parameters

were within the optimal conditions for the growth of *S. cerevisiae*. However, this yeast has a preference for glucose as the supplied energy source, and when supplied in a mixed growth medium *e.g.* brewers wort, it is the first sugar used (Lagunas, 1993). However, when glucose was supplied as the sole energy source the growth of the yeast reached maximum cell density at $69.25x10^6$ cells/ml (this was when the initial zinc concentration was supplied at 25.6μg/ml). Cell number assessments over the 4 initial zinc concentrations (which ranged from 0-3.2μg/ml) saw a general trend of increased cell number at 24 hours as the initial zinc concentration increased, followed by a decrease in cell growth, then cell density peaked at 25.6 μg/ml. Stewart and Russell (1998) have stated that an increase in the zinc concentration will lead to depressed cell growth in zinc concentrations above 0.6μg/ml, unless in similarly high concentrations of manganese (Hammond, 2000). When the media was unsupplemented with zinc, the growth of the lager yeast was suppressed (final cell density of $19.44x10^6$ cells/ml). Industrial brewers wort usually contains zinc in the range of 0.1-0.5 μg/ml (Stewart and Russell, 1998), if the wort is deficient in zinc then lager cells opt out of the cell division cycle and rest in the G_0 phase, and brewers report a "stuck" fermentation (Mochaba *et. al.* 1996). The overall performance of the lager yeast, when comparing growth with different carbohydrates was optimal when sucrose was supplied. The maximal growth achieved was $70.75x10^6$ cells/ml, when zinc was supplied at a level of 6.4μg/ml. With fructose, cell density peaked at $64.25x10^6$ cells/ml, and with maltose $41.5x10^6$ cells/ml, when the initial zinc concentration was 6.4 and 12.8μg/ml, respectively.

There appears to be some influence of zinc on the overall growth rates of the yeast, which has resulted in different growth patterns dependent upon the carbon source. When there was no zinc supplied, the growth of the lager yeast was generally unaffected during the 24 hour period examined, with the exception of when glucose was the supplied metabolisable energy source. However, the effect that this nutrient limitation would have had on the cell

population over a longer period of time may have been detrimental, with depressed cell growth and protein production inhibited (Obata *et. al.*, 1996). Maskell *et. al.*(2001) have reported on the impact of carbohydrate on the replicative lifespan of lager yeast. Their findings showed that carbohydrate availability may influence the lifespan of lager yeast, which in turn is directly proportionate to the growth of the yeast.

Therefore, in conclusion, the carbohydrate supplied can influence the growth rate of a lager strain of *S. cerevisiae* as can the initial zinc concentration supplied in the YPDM, with maximum growth achieved when sucrose was the supplied energy source and the initial zinc concentration was 6.4 μg/ml.

Zinc accumulation by lager brewing yeast

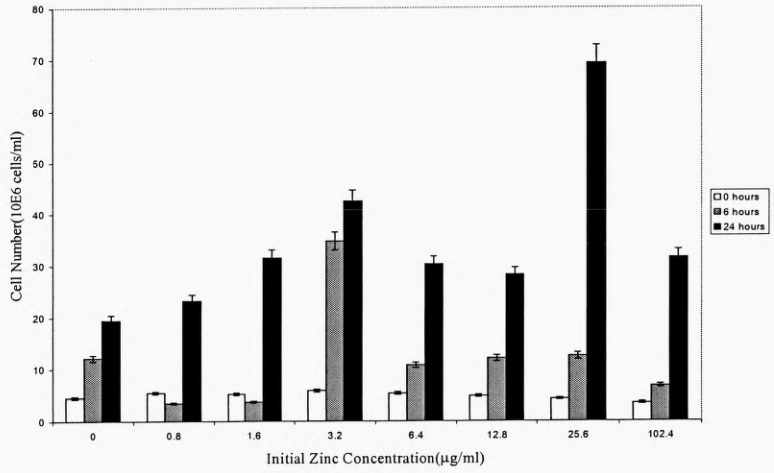
When examining the zinc accumulating ability of a strain of lager yeast, which was grown in YPDM, containing different sugars as the metabolisable energy source; glucose (Figure 4.3.4.1), fructose (Figure 4.3.4.2), maltose (Figure 4.3.4.3) and sucrose (Figure 4.3.4.4). Maltose supplemented media stimulated maximal uptake at time 0 and 6 hours, in comparison to the other sugars studied. The maximum biosorptive effect (time 0) demonstrated by this lager strain when expressed on a per cell basis was 693.9 fg zinc/cell, this is equivalent to 3.183 μg/ml (data not shown) of zinc uptake by the total population of the cells. The 0 time results when the yeast was supplied with the YPDM which was supplemented with :glucose was 408.3 fg zinc/cell (equivalent to 1.429 μg/ml); fructose 473.1 fg zinc/cell (2.933 μg/ml) and; sucrose 143.6 fg zinc/cell (0.783 μg/ml). These maximal amounts of zinc removed from the media, which was due to the physical effect of biosorption, was achieved when the media was supplemented with the highest initial zinc concentration (102.4 μg/ml).

After a period of 6 hours, the amount of zinc accumulated by the lager yeast was 2353 fg zinc/cell (when the media was supplemented with maltose as the metabolisable energy source, the total zinc accumulated by the cell population was 23.06 μg/ml), glucose; 374.5 fg zinc/cell (total zinc accumulated; 2.346 μg/ml) fructose; 988.5 fg zinc/cell (total zinc accumulated; 10.32 μg/ml) and sucrose; 549.9 fg zinc/cell (total zinc accumulated; 7.96μg/ml). The maximum levels of zinc sequestered were all from the maximum initial zinc concentration of 102.4 μg/ml, with the exception of the cells which were grown in the presence of fructose. These cells achieved maximum uptake as a cell population of 11.35 μg/ml, from an initial zinc concentration of 25.6 μg/ml, this uptake was approximately 45% of the available zinc supplied in the media. The 24 hours results which relate to zinc accumulation, when expressed as fg zinc/ cell, was optimal uptake levels of 430.5 fg zinc/cell when YPDM was supplemented with fructose. When expressing this result as zinc uptake per total cell population (16.36 μg/ml), the cells accumulated a further 6.04 μg/ml of zinc since 6 h. The maximal uptake rates achieved by the lager yeast when the cells were grown in the media supplemented with the other carbon sources were: 372.6 fg zinc/cell (maltose); 290.8 fg zinc/cell (sucrose); and 120.7 fg zinc/cell (glucose).

In comparing the results, maltose stimulated greater accumulation over the initial time periods examined (0 and 6 hours). Maltose is the predominant sugar in brewers wort, originating from the cereal crops that the lager yeast ferments. Therefore, this yeast obtained maximal sequestration of zinc with the industrial substrate of choice during the initial experimental time course. However, after 24hours, intracellular accumulation was maximal when the monosaccharide fructose was the supplied carbohydrate.

Figure 4.3.4.1: **Interactions between zinc ions and *S. cerevisiae* lager yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained glucose as the metabolisable energy source.

a)



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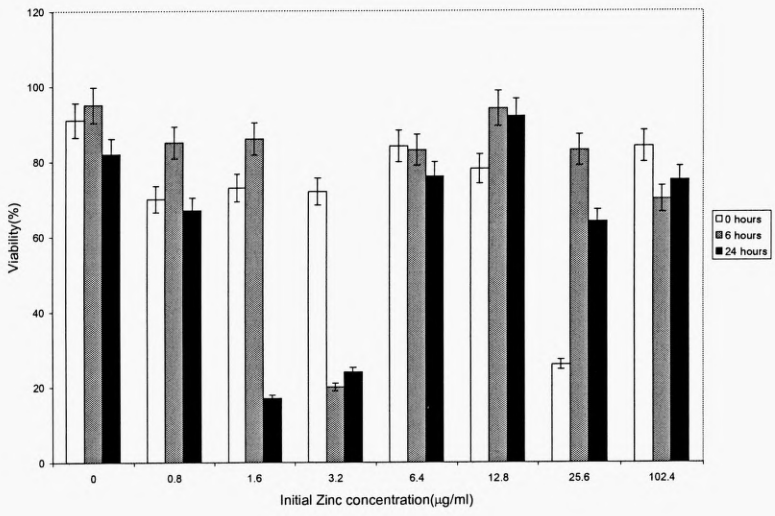

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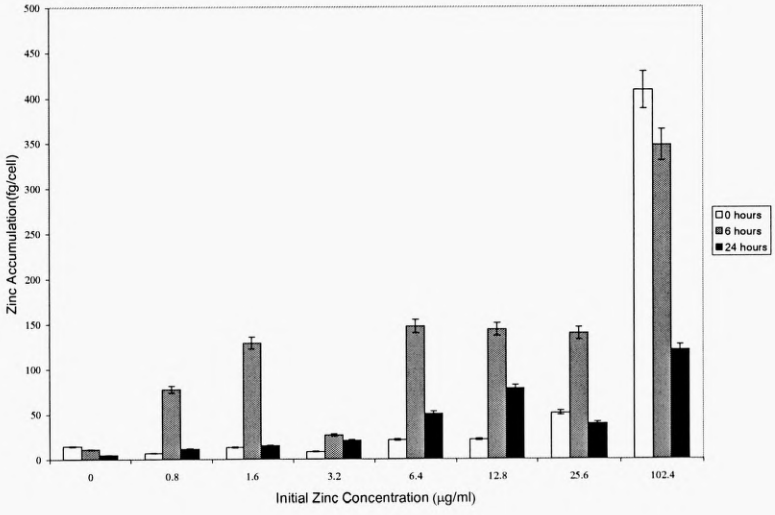
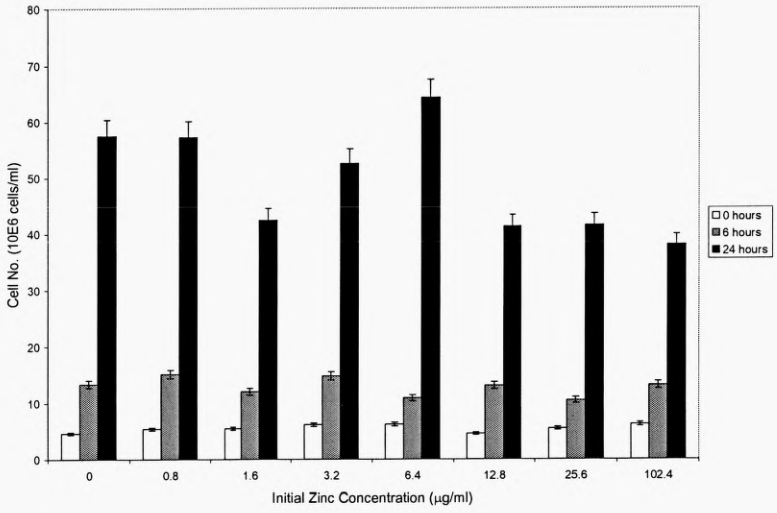


Figure 4.3.4.2: **Interactions between zinc ions and *S. cerevisiae* lager yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained fructose as the metabolisable energy source.

a)



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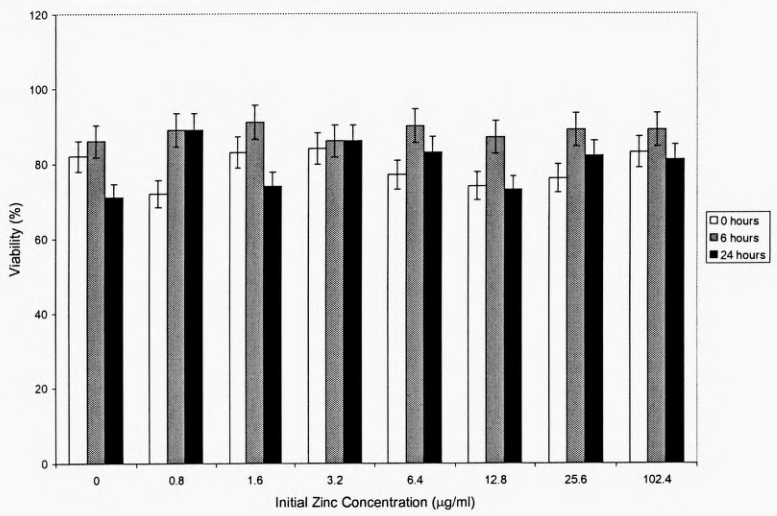

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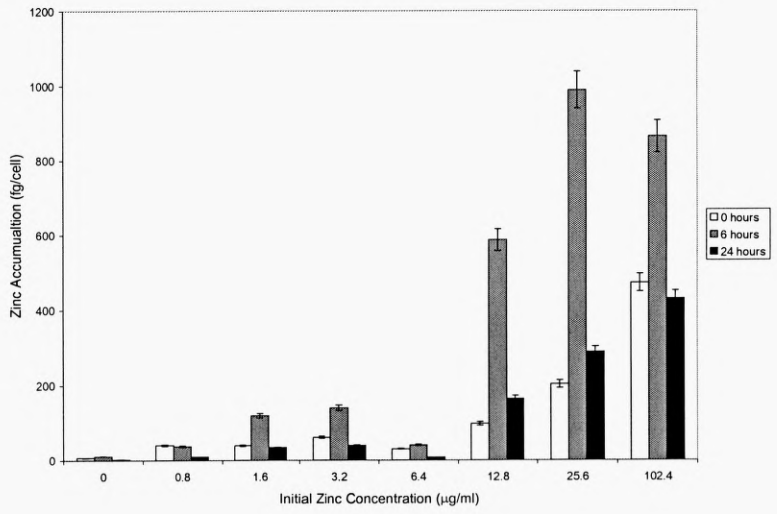
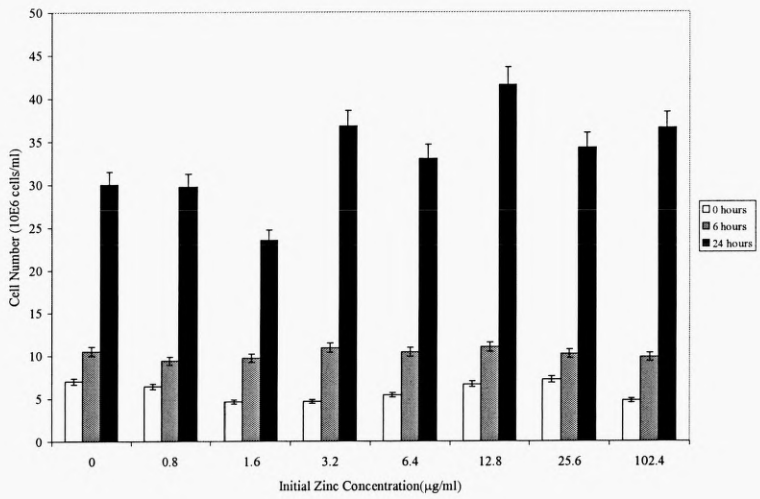


Figure 4.3.4.3: **Interactions between zinc ions and *S. cerevisiae* lager yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained maltose as the metabolisable energy source.

a)



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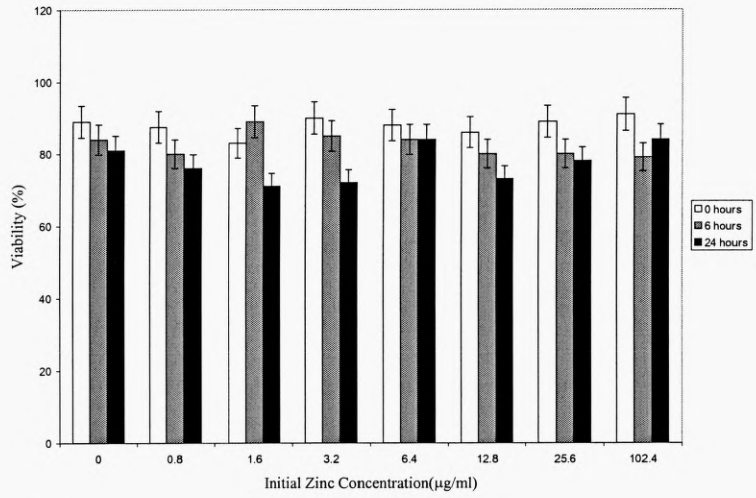

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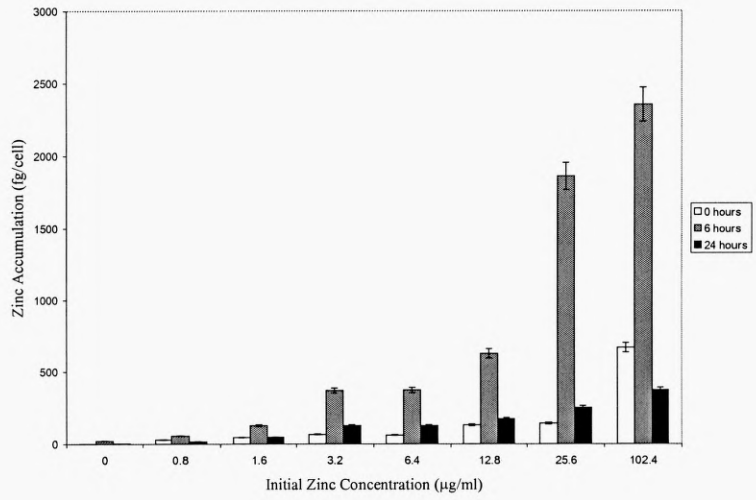
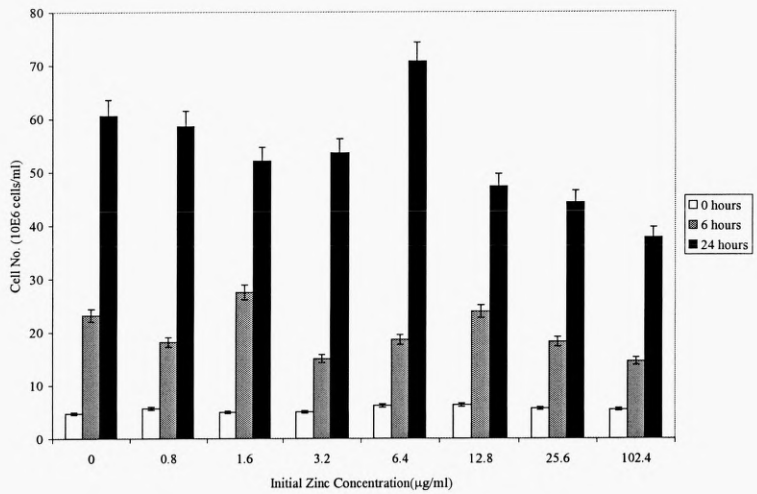


Figure 4.3.4.4: **Interactions between zinc ions and *S. cerevisiae* lager yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained sucrose as the metabolisable energy source.

a)



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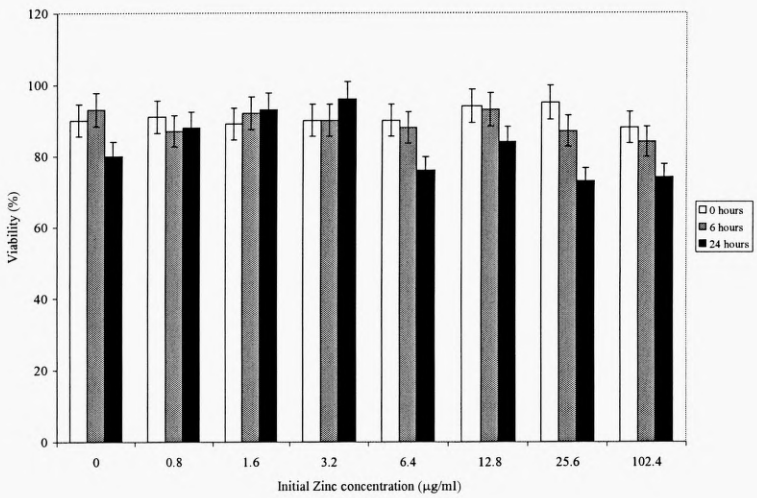

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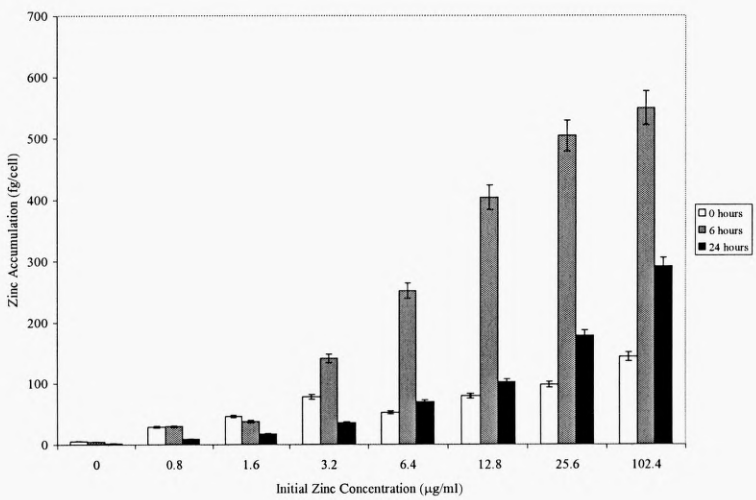


4.3.5 *S. cerevisiae* distillers yeast.

The ability of distillers yeast to grow when supplied with different carbohydrates is shown in Figures 4.3.5.1a, 4.3.5.2a, 4.3.5.2a and 4.3.6.2a (glucose, fructose, maltose and sucrose respectively). This yeast grew extremely well under all the growth parameters examined. Distillers yeast maximal growth was achieved when the supplied energy source was fructose, with growth reaching $130x10^6$ cells/ml, when the media contained no supplemented zinc. The results for the other three carbohydrates examined demonstrated maximal growth in the unsupplemented media (glucose $127.25x10^6$ cells/ml and sucrose $120x10^6$ cells/ml). When the distillers yeast was grown in the presence of maltose ($64.5x10^6$ cells/ml), with maximal growth rate achieved after the examined 24 hour period when the media was supplemented with 3.2μg/ml, however when statistically examining the growth rates at the 5% level of significance it was determined that the growth rates were not significantly different over the initial zinc concentration gradient.

This rather strange result may indicate that distillers yeasts affinity and necessity for biologically available zinc may not be as great as the other yeasts examined. Distillers yeast is grown industrially on molasses. This industrial growth substrate is unsupplemented with zinc, therefore this strain of distillers yeast may have adapted to grow in media that may be exceptionally low and possibly deficient in essential nutrients.

Zinc accumulation by distillers yeast

The accumulation of zinc by distillers yeast which has been grown in different metabolisable energy sources was optimal when maltose was the supplied energy source at time 0 and 6 hours. This pattern of accumulation was displayed throughout these time intervals with maximum sequestration rate reaching 1056.85fg zinc/cell at 0 time. This was equivalent to 8.719 μg/ml of zinc accumulated per population (Figure 4.3.5.3c). This extremely high level of uptake was at 0 time, therefore, it may be assumed that this rate is

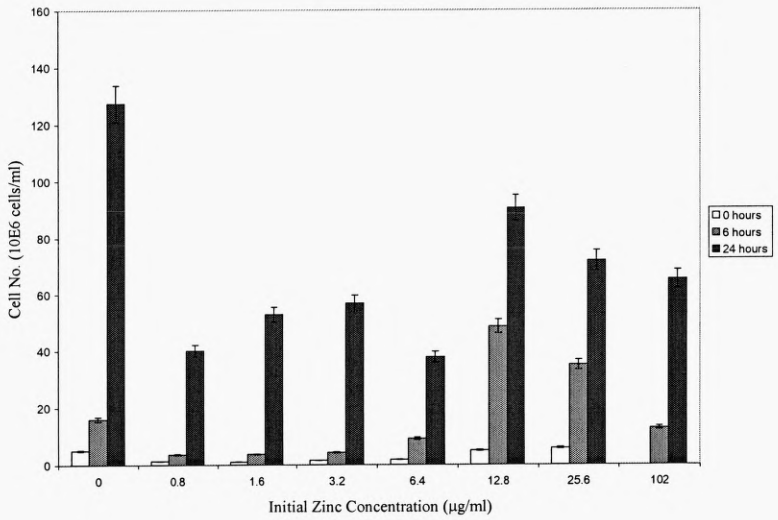
due to the physical effect of biosorption. The Zn uptake levels when glucose was supplied as the energy source was reached a maximum of 226.5fg zinc/cell (equivalent to 0.77 μg/ml)(Figure 4.3.5.1c), again at time zero. At 0 hours the zinc levels accumulated by the distillers yeast when grown in the presence of fructose reached 218.7 fg zinc/cell (0.634 μg/ml) (Figure 4.3.5.2) and when sucrose was the supplied energy source the distillers yeast sequestered 197.1 fg zinc/cell (1.124 μg/ml) (Figure 4.3.5.4). Maximum uptake, was achieved when the distillers yeast was utilising the disaccharide maltose as the metabolisable energy source and after 6 hours zinc uptake reached 583.2 fg zinc/cell (7.64 μg/ml). The zinc accumulation ability of the distillers yeast when grown in the presence of the other sugars demonstrated with glucose based YPDM uptake reached 149.5 fg zinc/cell, fructose stimulated 218 fg zinc/cell and sucrose stimulated 393 fg zinc/cell.

However, upon examination of the 24 hours result, maximum zinc accumulation occurred with the disaccharide sucrose, which stimulated 11.2 μg/ml per culture population from an initial concentration of 102.4 μg/ml. When expressed on a per cell basis, results show that glucose grown cells contained more zinc (198 fg zinc/cell), compared with the sucrose grown cells (161 fg zinc/cell). However, distillers yeast generally accumulated more zinc from media which had been supplemented with the highest amounts of zinc (102.4μg/ml).

Zinc accumulation by distillers yeast was, therefore, influenced by time and the type of metabolisable energy source supplied. Distillers yeast generally ferments cereal based extracts. Therefore, the industrial based carbohydrate from this crop (maltose), stimulated maximum zinc accumulation. However, after 24hours, when the cells had been accumulating zinc intracellularly, the maximum zinc uptake was achieved by the sucrose or glucose, depending on which expression method was examined. The sucrose which would have been broken down extracellularly by invertase into glucose and fructose, (yields the lowest value) implying that the monosaccharides stimulate greatest zinc accumulation.

Figure 4.3.5.1: **Interactions between zinc ions and *S. cerevisiae* distillers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained glucose as the metabolisable energy source.

a)



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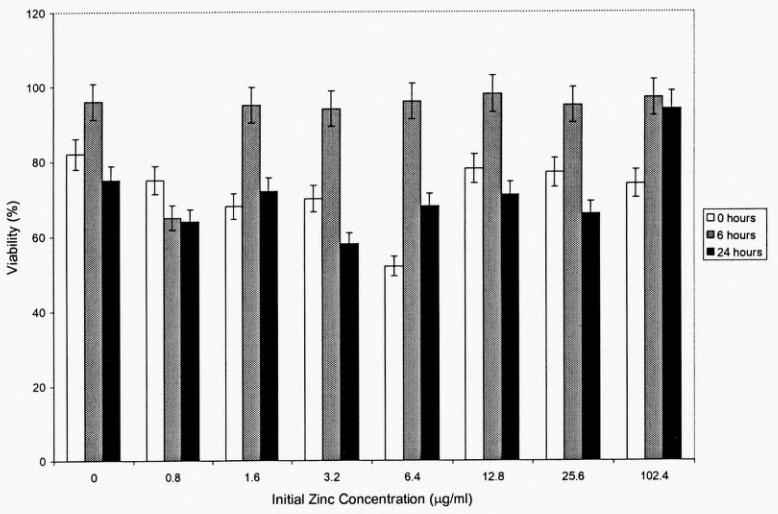

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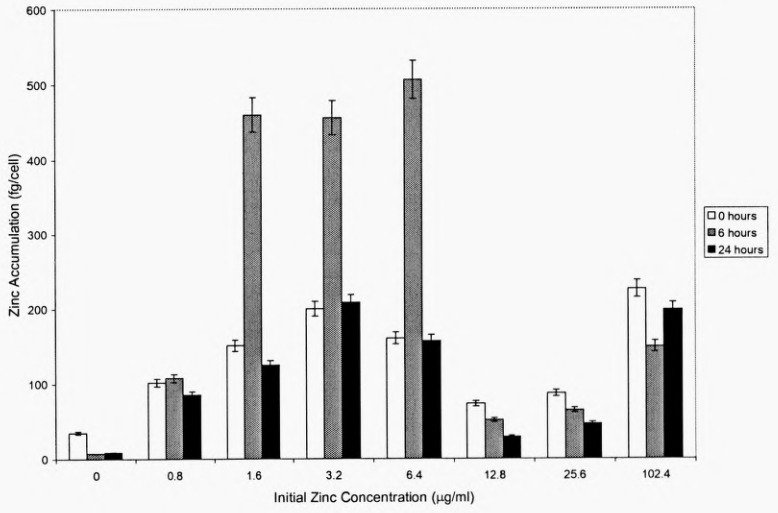
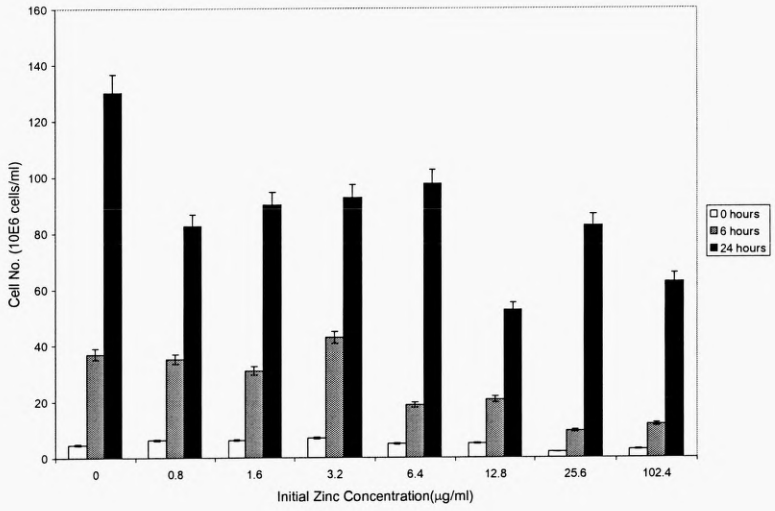


Figure 4.3.5.2: **Interactions between zinc ions and *S. cerevisiae* distillers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained fructose as the metabolisable energy source.

a)



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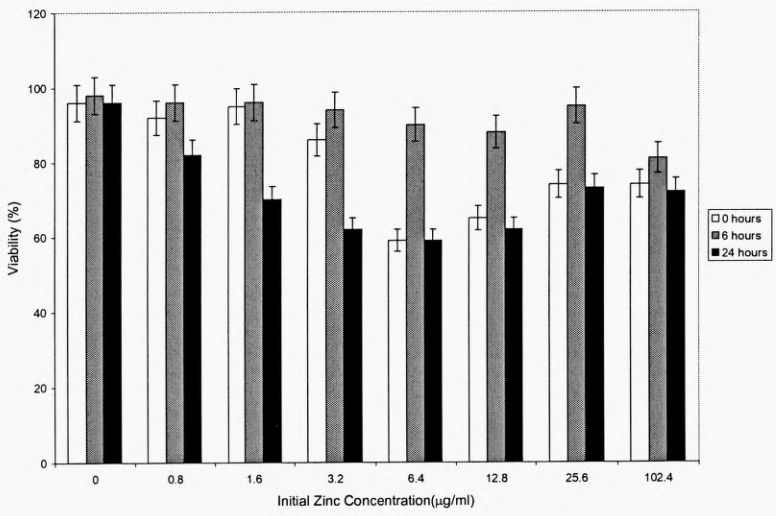

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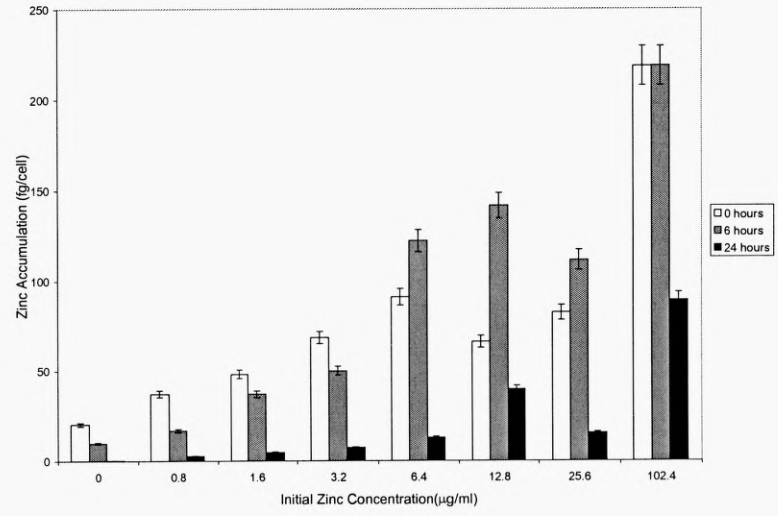
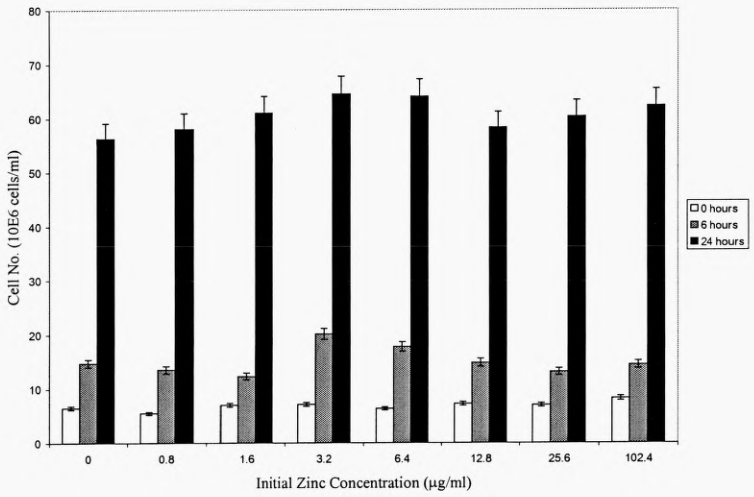


Figure 4.3.5.3: **Interactions between zinc ions and *S. cerevisiae* distillers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained maltose as the metabolisable energy source.

a)



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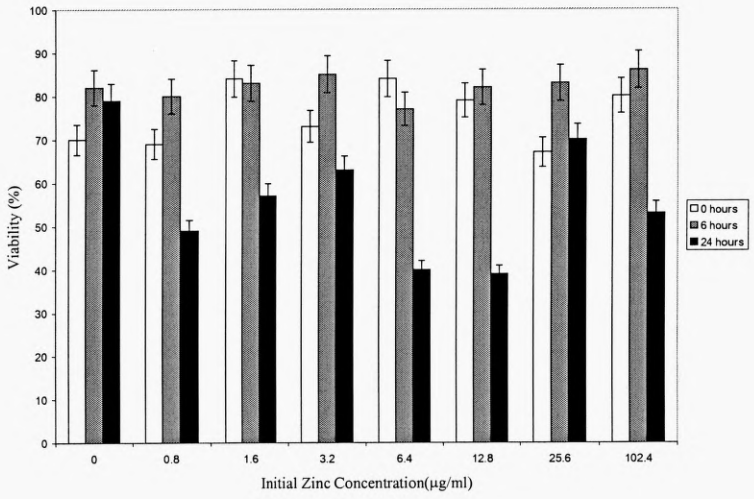

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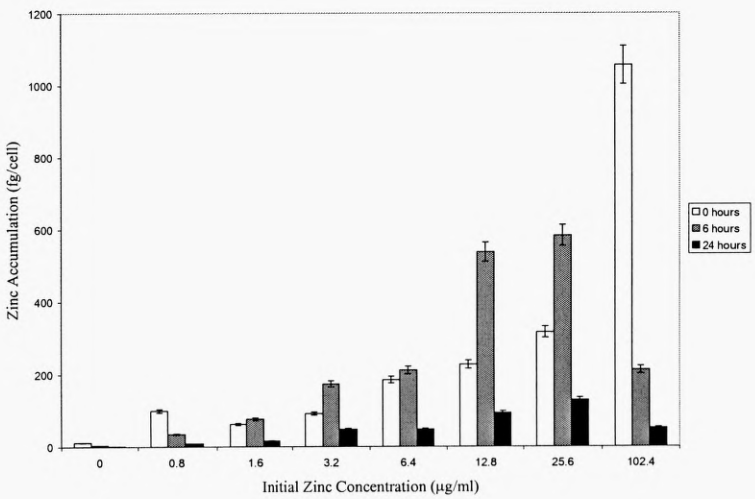
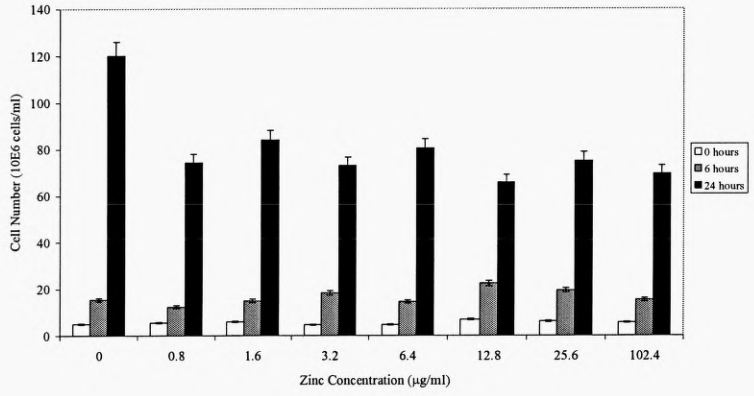


Figure 4.3.5.4: **Interactions between zinc ions and *S. cerevisiae* distillers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained sucrose as the metabolisable energy source.

a)



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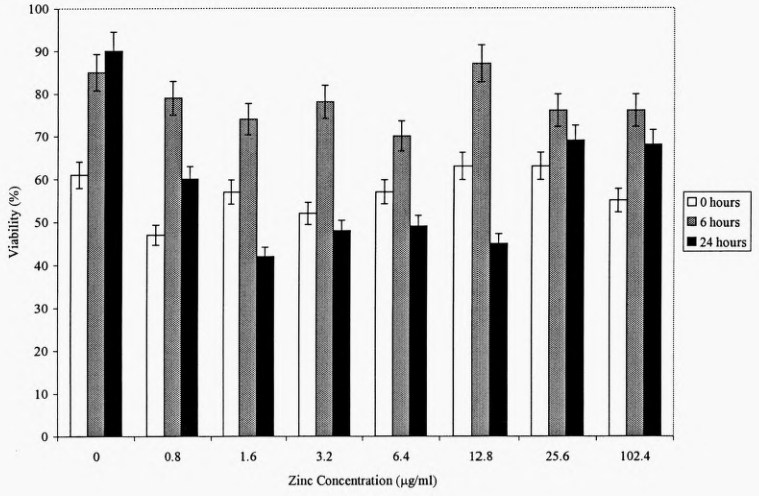

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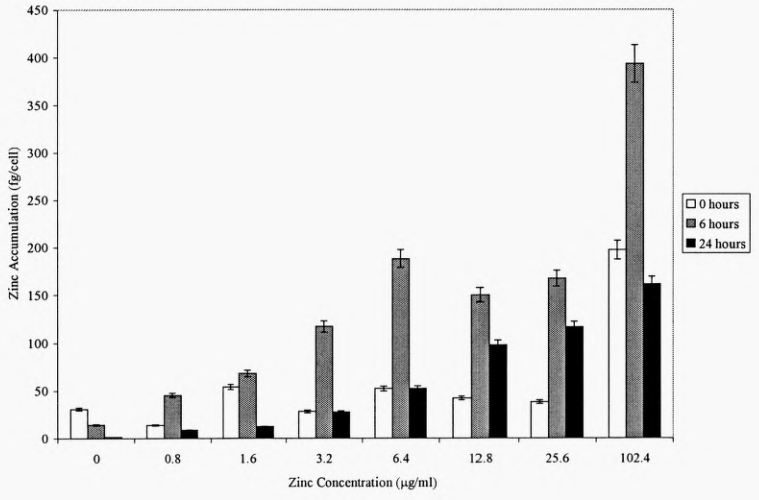


4.3.6 *S. cerevisiae* wine yeast.

Wine yeast is usually supplied with grape juice as the common carbon source, the main sugars of which are fructose and glucose, with a trace of sucrose (Rainieri and Pretorius, 2000). Therefore, wine yeasts normally metabolise the energy sources examined with the exception of maltose. The ability of wine yeast to grow when supplied with the different carbohydrates is shown in Figures (4.3.6.1a, 4.3.6.2a, 4.3.6.3a and 4.3.6.4a: glucose, fructose, maltose and sucrose respectively). This yeast grew exceptionally well especially when fructose and sucrose were the supplied carbohydrates. The wine yeast maximal growth achieved was $270x10^6$ cells/ml with sucrose, when the media contained 102.4 μg/ml zinc. The results for the other three carbohydrates examined demonstrated maximal growth in zinc supplemented media, with the following maximal cell densities achieved: glucose, $144.75x10^6$ cells/ml (with 0.8μg/ml Zn); fructose, $167.25x10^6$ cells/ml (with 0.8μg/ml Zn); maltose, $133.5x10^6$ cells/ml (with 12.8μg/ml Zn) after 24 h. The results were as expected for glucose and fructose, that is, high growth rates for normally metabolised energy sources. However, since the sucrose would be broken down into its component monosaccharides outside the cell by the enzyme invertase, this may account for the high growth rates with the yeast preferentially utilising the glucose first, then the fructose. The growth of the wine yeast on the disaccharide was greater than the growth of this species on the individual sugars.

The maximal growth rates achieved in the defined media when containing the monosaccharides glucose and fructose was at the low end of the zinc concentration gradient (0.8 μg/ml). The cell densities achieved when the wine yeast was grown in the presence of the disaccharides, maltose and sucrose were at the higher end of the zinc concentration gradient. This demonstrates that for the maximal growth of wine yeast in the presence of different metabolisable energy source that different concentrations of zinc are required.

Industrial based media for the growth of wine yeast is grape must, this media is rich in monosaccharides, therefore, if this was also to be used for the propagation of the yeasts then it would be advisable to determine the amount of zinc in the must. Excessive zinc appears to have a detrimental effect on the cell densities in the wine yeast (when grown in glucose based YPDM) when the initial zinc concentration was greater than 1.6μg/ml. The cell densities increases again at 12.8 μg/ml and 102.4 μg/ml Zn, this may be due to these excessive concentrations having a depressed effect on cell growth. Hammond (2000) reported that excessive zinc could depress the growth of lager yeast unless the concentrations of manganese were similarly high.

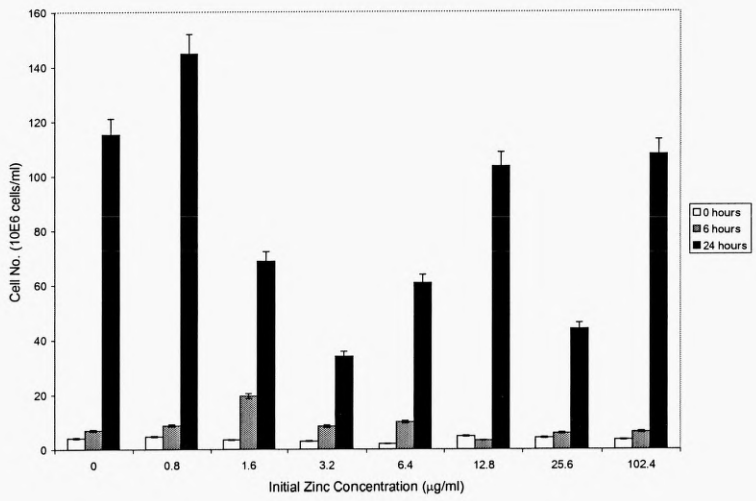
Zinc accumulation by wine yeast

Upon examination of the influence of the metabolisable energy source on zinc accumulation by wine yeast, throughout the time intervals examined (0 and 24 hours), it was found that fructose based YPDM stimulated the greatest amount of zinc acquisition (Figure 4.3.6.2c). However, at 6 hours, glucose based YPDM stimulated greatest uptake (Figure 4.3.6.1c). This pattern of zinc uptake was represented when the data was expressed on a per cell basis (fg zinc/cell) or as total zinc accumulated (μg/ml), with uptake reaching 277.6 fg zinc/cell at time 0, 412 fg zinc/cell at 6 hours and 61.16 fg zinc/cell at 24 hours. The maximum biosorptive effect the wine yeast demonstrated with the other carbohydrates was; 105.7 (glucose), 14.3 fg zinc/cell (maltose)(Figure 4.3.6.3c) and 73.1 (sucrose) (Figure 4.3.6.4c). After 24 hours, the maximum Zn accumulation was achieved by the wine yeast in fructose based YPDM. When grown in YPDM containing sucrose wine yeast allowed sequestered 46 fg zinc/cell, 46.2 fg zinc/cell (with glucose) and 29.3 fg zinc/cell (with maltose). The pattern of zinc uptake by wine yeast was optimal with monosaccharides (glucose and fructose) in comparison to the disaccharides studies. Wine yeast is generally grown in a

grape must, which consists of fructose, glucose and sucrose and the accumulation of zinc in these carbon sources was higher than when the cells were supplied with maltose (which is absent in grape must) as the metabolisable energy source. In supplementing the YPDM with maltose, the growth rate and the zinc accumulation rates were effected, in comparison to the other sugars.

Figure 4.3.6.1: **Interactions between zinc ions and *S. cerevisiae* wine yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained glucose as the metabolisable energy source.

a)



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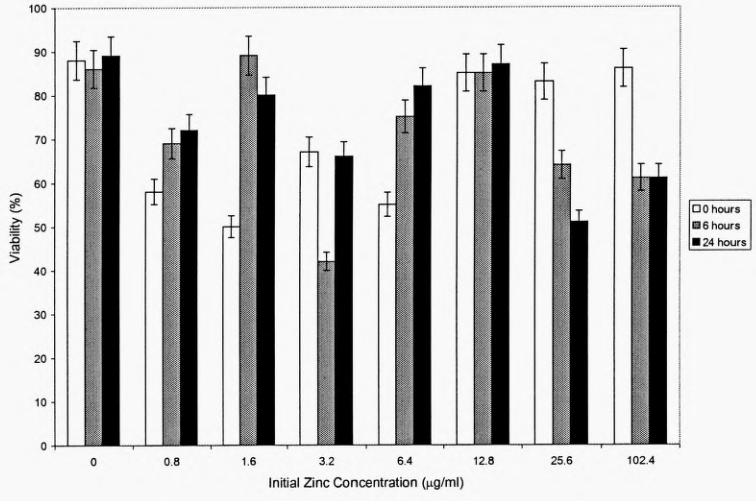

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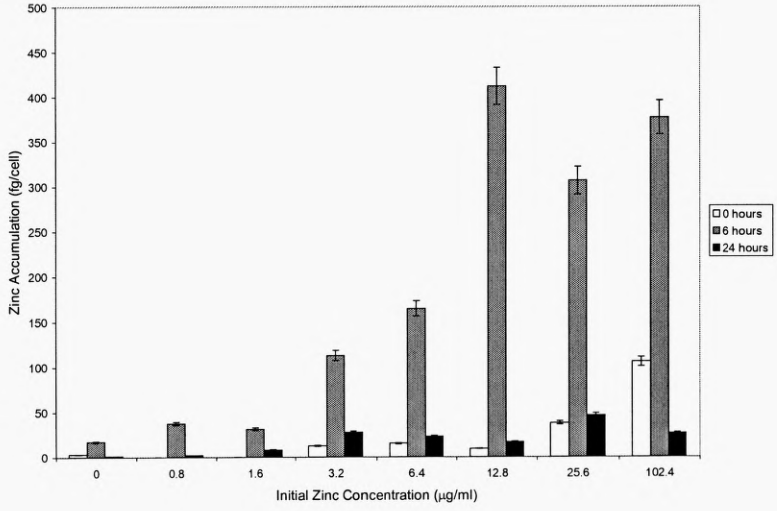
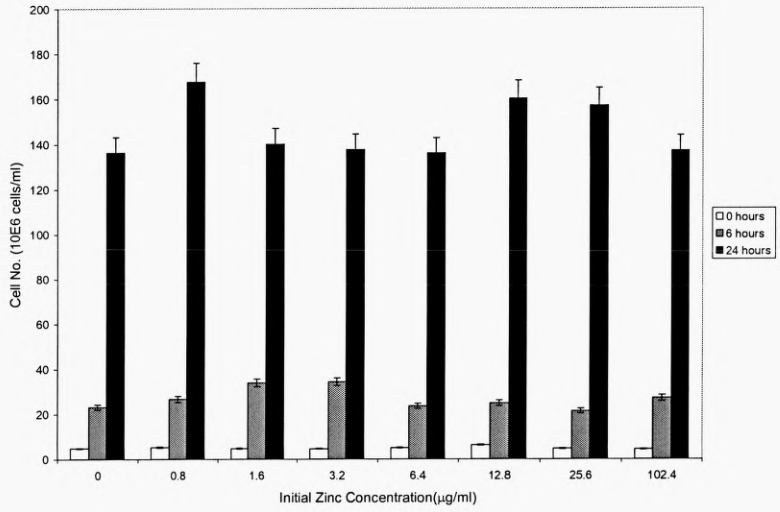


Figure 4.3.6.2: **Interactions between zinc ions and *S. cerevisiae* wine yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained fructose as the metabolisable energy source.

a)



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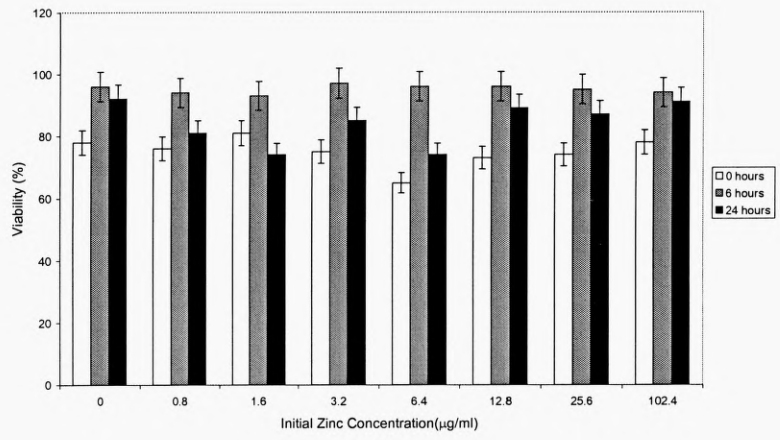

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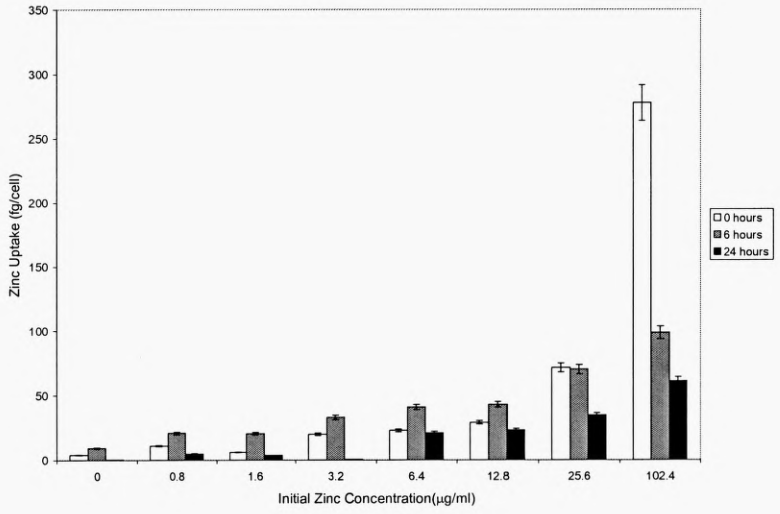
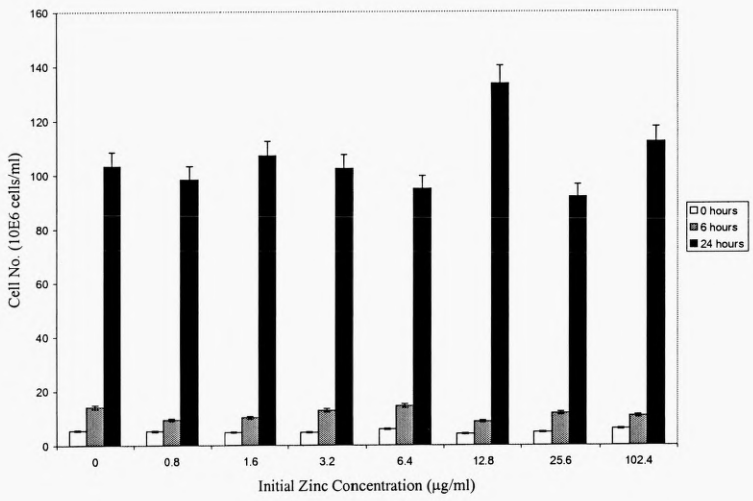


Figure 4.3.6.3: **Interactions between zinc ions and *S. cerevisiae* wine yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained maltose as the metabolisable energy source.

a)



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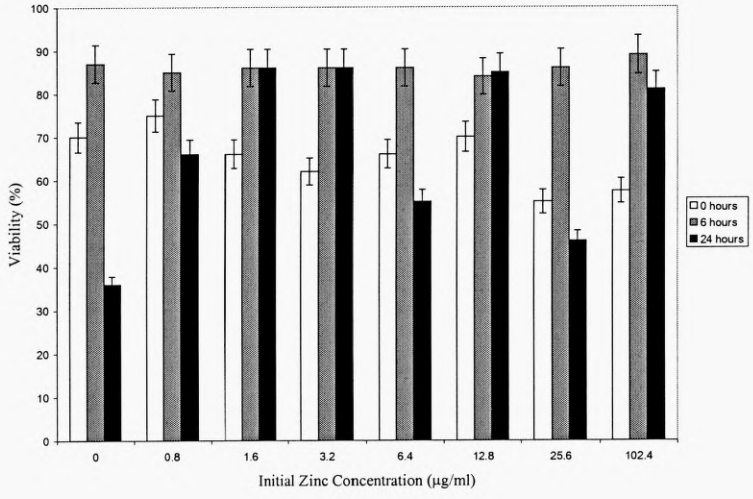

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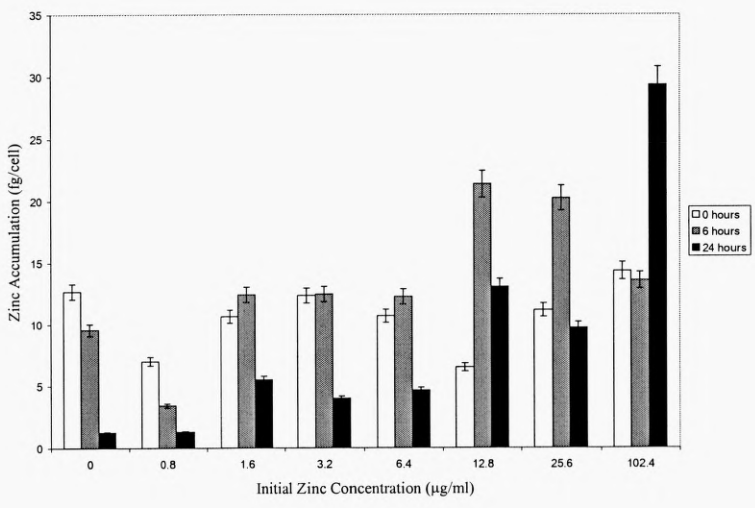
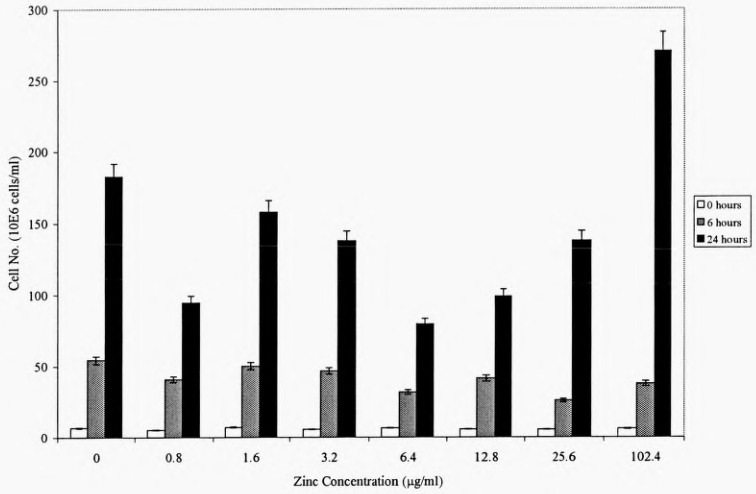


Figure 4.3.6.4: **Interactions between zinc ions and *S. cerevisiae* wine yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained sucrose as the metabolisable energy source.

a)



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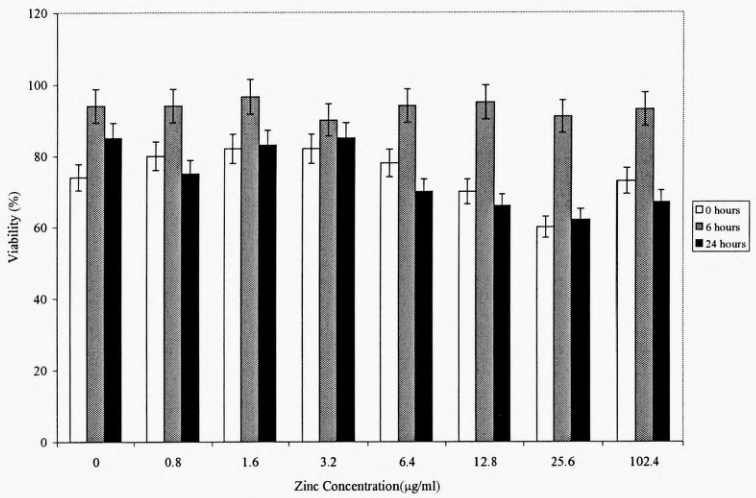

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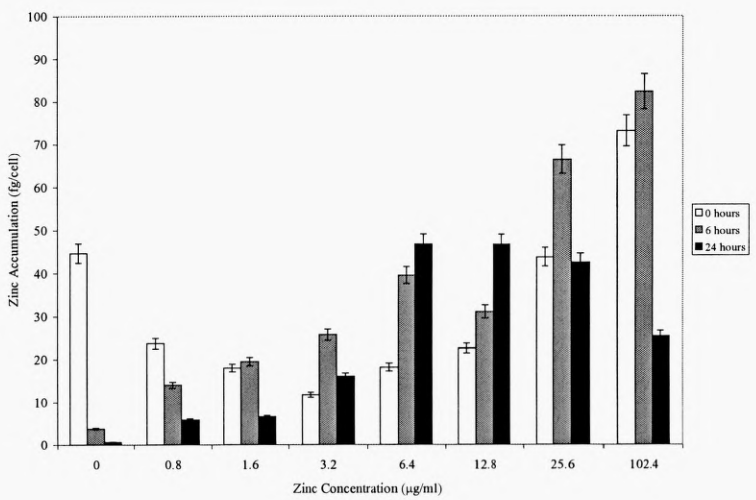


4.3.7 *S. cerevisiae* bakers yeast.

Bakers yeast is propagated on a sucrose rich medium (molasses), and is utilised in the leavening of maltose rich dough (Walker, 1998a). The maximal growth of the experimental cultures were fairly evenly distributed, with all the carbohydrates showing various changes in the overall growth rates, with respect to the initial zinc concentration. The ability of bakers yeast to grow when supplied with different carbohydrates is shown in Figures 4.3.7.1a, 4.3.7.2a, 4.3.7.3a and 4.3.7.4a (glucose, fructose, maltose and sucrose respectively). The optimal growth was 90.75×10^6 cells/ml final cell density, when the culture was grown in unsupplemented zinc based media when fructose was the supplied metabolisable energy source. With maximal growth achieved by the bakers yeast when grown in a minimal media containing glucose 70.25×10^6 cells/ml (0μg/ml zinc supplemented); maltose, 73.25×10^6 cells/ml (1.6 μg/ml zinc supplemented) and sucrose, 76.8×10^6 cells/ml (102.4μg/ml zinc supplemented). Upon examination of the viability of the bakers yeast (Figure 4.3.7.1 b), demonstrated that after a period of 6 hours the bakers yeast grown in the presence of 102.4 μg zinc/ml, the viability represented was rather low, this may be due to experimental error. The culture viability after 24 hours was approximately 80%, thus demonstrating a relatively healthy population of cells.

Zinc accumulation by bakers yeast

Zinc uptake by bakers yeast grown in different metabolisable energy sources, was maximal when the cells were supplied with glucose over the initial time intervals (0 and 6 hours, 554.6 fg zinc/cell and 620 fg zinc/cell, respectively) (Figure 4.3.7.1c). The presence of other sugars stimulated zinc uptake, with maltose stimulating the least uptake (Figure 4.3.7.3c). The results from the individual sugars demonstrated initial uptake levels (0 time) of 420 fg zinc/cell (with fructose); 172.5 fg zinc/cell (with sucrose) and 85.2 fg zinc/cell

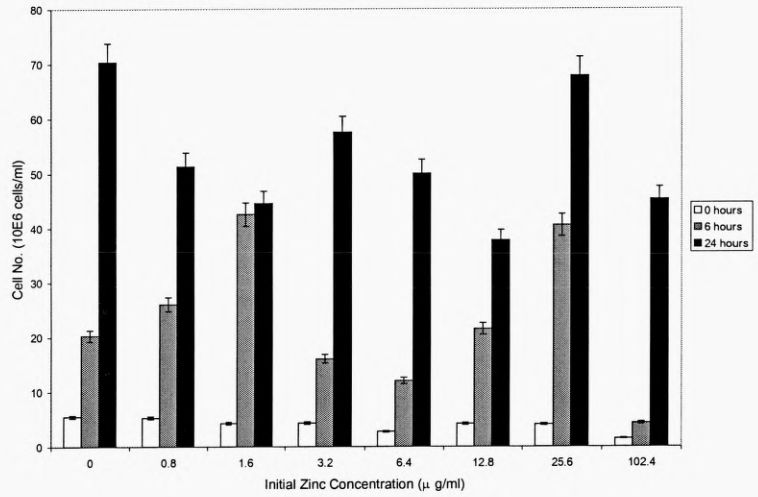
(with maltose). The uptake after 6 hours was 289 fg zinc/cell (with fructose); 550 fg zinc/cell (with sucrose) and 108 fg zinc/cell (with maltose).

The maximum bioaccumulative effect (after a period of 24 hours) was achieved when the cells were grown in fructose based media (figure 4.3.7.2c), the cells achieved an intracellular level of 183.3 fg zinc/cell. When bakers yeast was grown in the presence of the other metabolisable energy sources, glucose stimulated 85.4 fg zinc/cell, maltose stimulated 168.6 fg zinc/cell and sucrose stimulated 137.6 fg zinc/cell. Although the levels of zinc accumulation by the bakers yeast when grown in YPDM containing fructose and maltose are similar, they are significantly different when tested at the 5% level.

Upon examination of these results obtained from the bakers yeast, the monosaccharides, glucose and fructose stimulated greater amounts of zinc uptake, over the time interval studied.

Figure 4.3.7.1: **Interactions between zinc ions and *S. cerevisiae* bakers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained glucose as the metabolisable energy source.

a)



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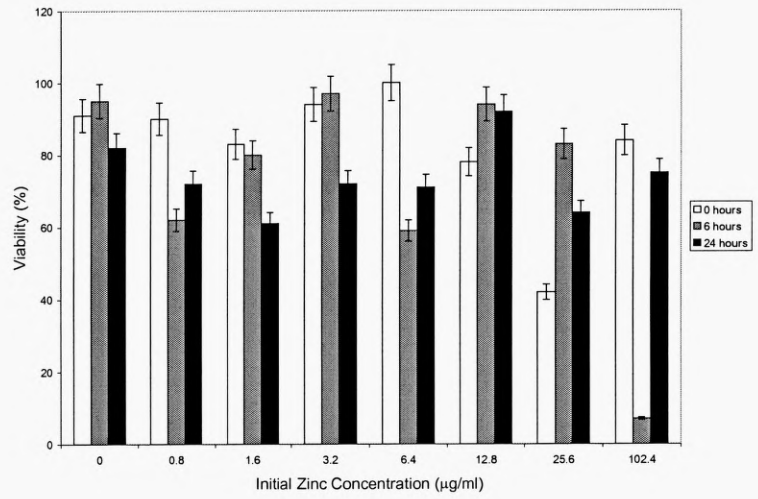


c)

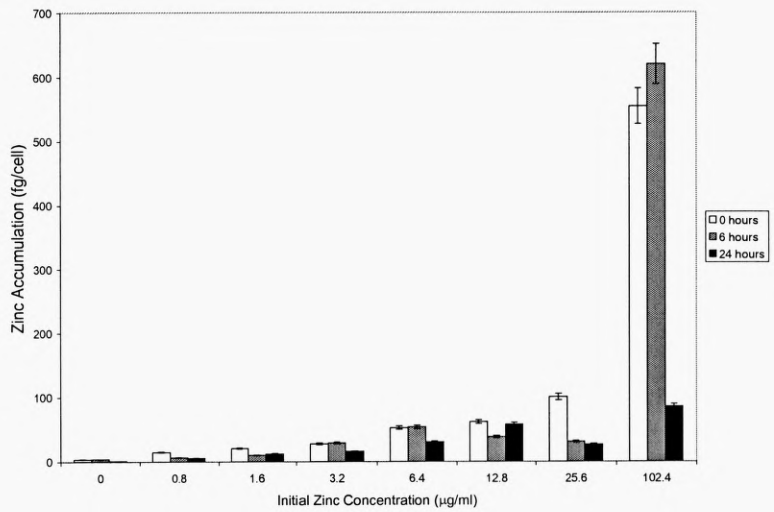
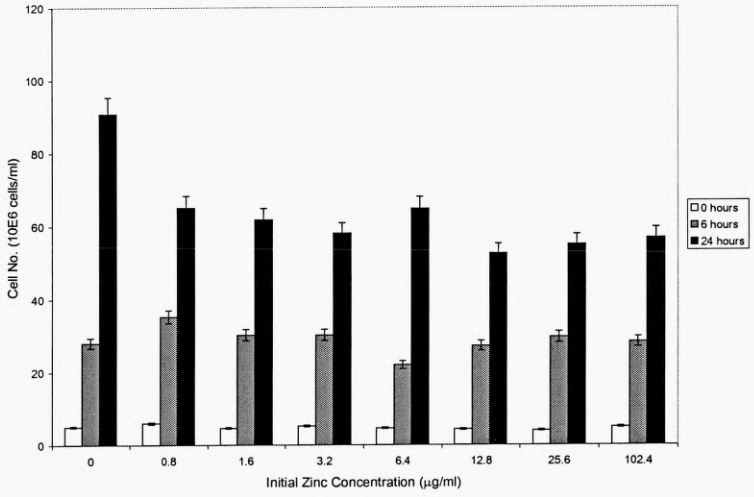


Figure 4.3.7.2: **Interactions between zinc ions and *S. cerevisiae* bakers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained fructose as the metabolisable energy source.

a)



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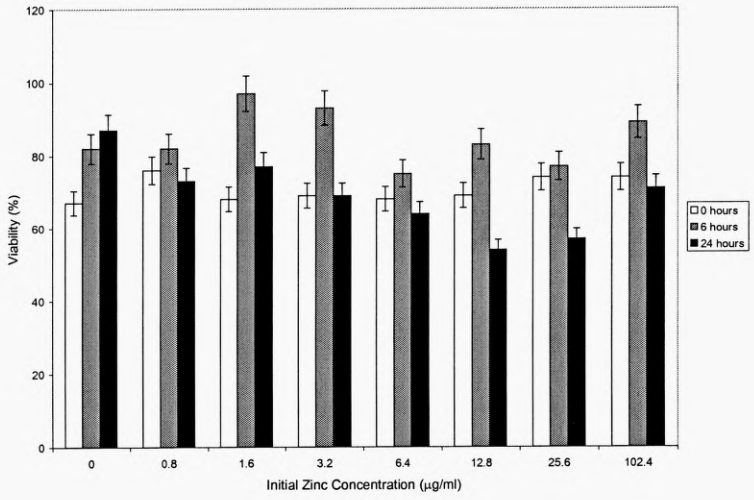

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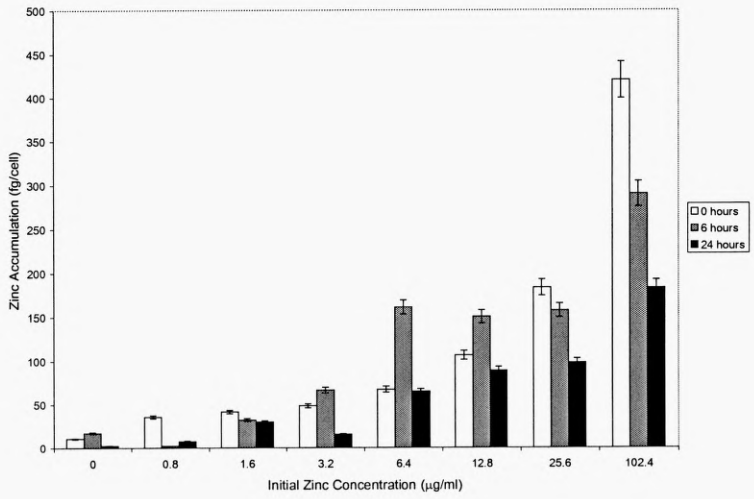
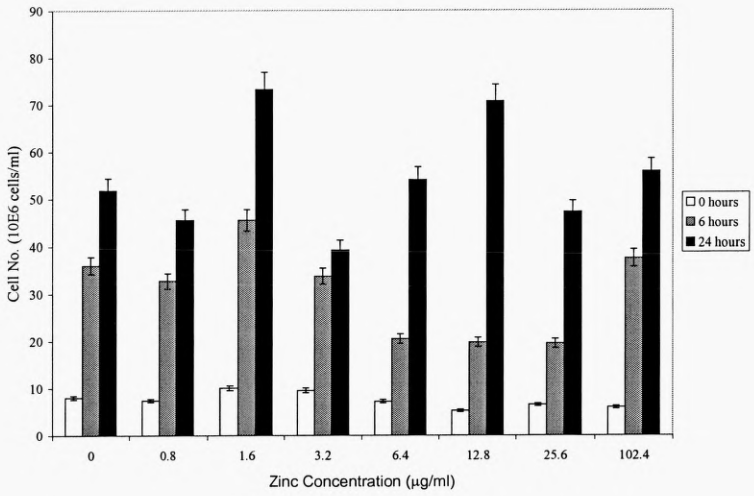


Figure 4.3.7.3: **Interactions between zinc ions and *S. cerevisiae* bakers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained maltose as the metabolisable energy source.

a)



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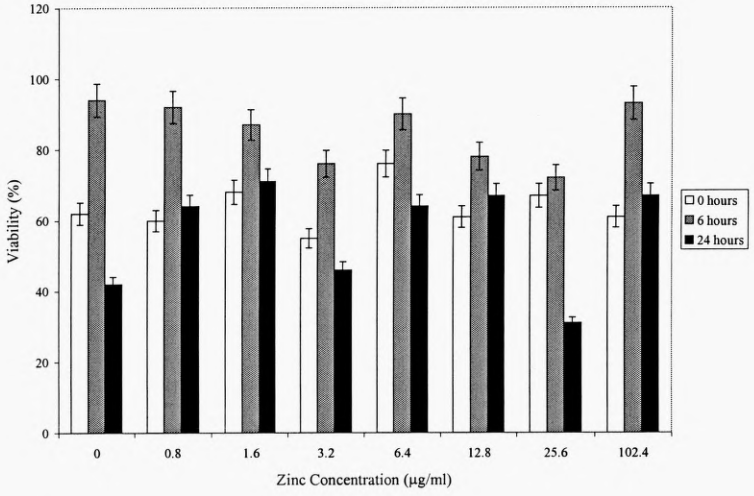

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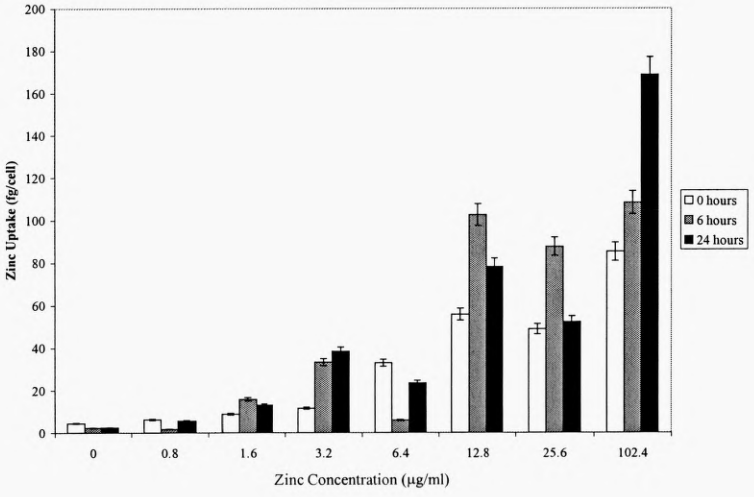
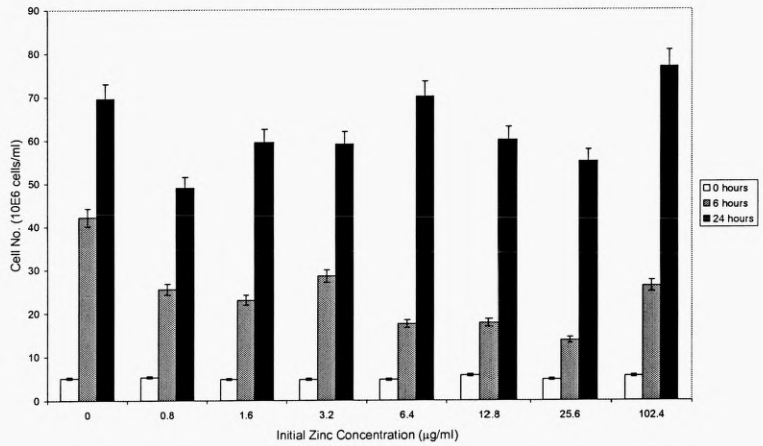


Figure 4.3.7.4: **Interactions between zinc ions and *S. cerevisiae* bakers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained sucrose as the metabolisable energy source.

a)



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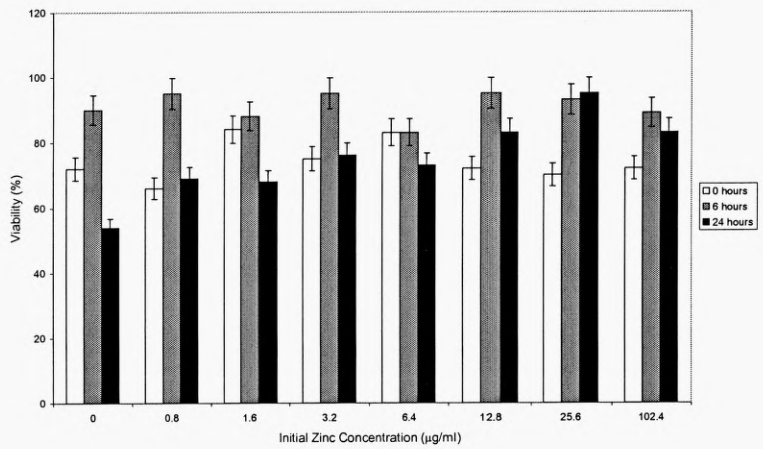

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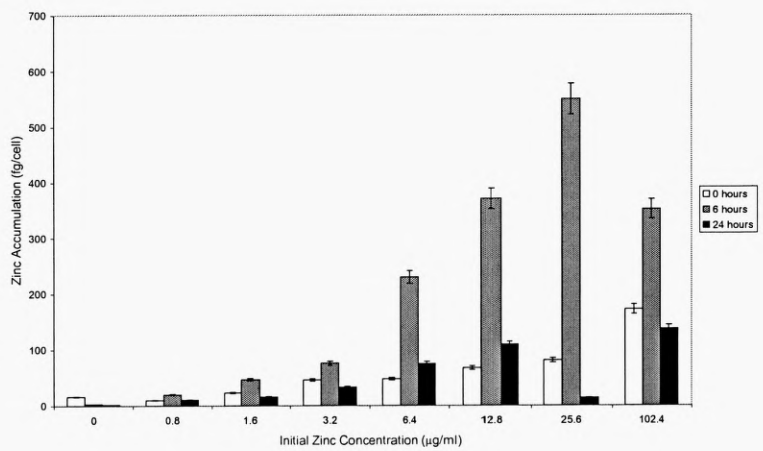


In conclusion, monosaccharides stimulated greater levels of zinc accumulation (after 24hours) in all the strains examined. Therefore, zinc accumulation was influenced by the chemical nature of the metabolisable energy source and there was also intra-strain differences in zinc uptake after 24 hours. When all the industrial yeasts were compared, lager yeast sequestered the most zinc after a 24 h period.

Table 5: Summary of zinc accumulation by industrial strains of *S. cerevisiae*

Strains	Maximum zinc accumulated (24hours) (fg zinc/cell)	Carbohydrate stimulating maximum accumulation
Lager	430	Fructose
Distillers	198	Glucose
Wine	61	Fructose
Bakers	183	Fructose

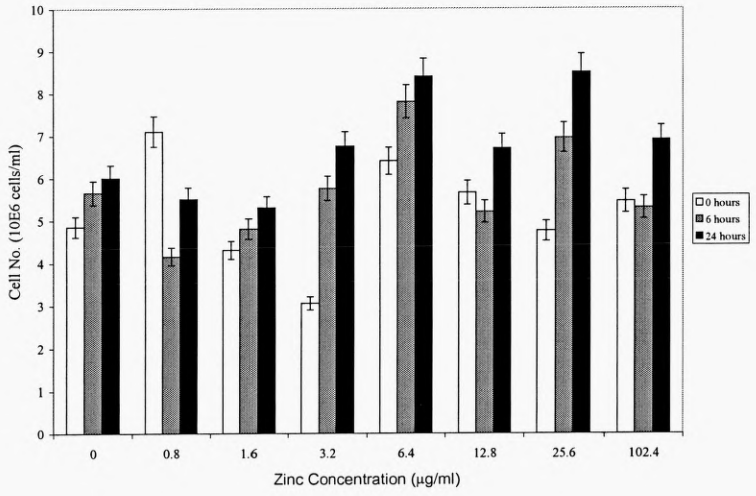
The enhanced stimulation of zinc uptake by monosaccharides, in comparison to the disaccharides may be due to the differences in the energy consumption by the cells. The monosaccharides were fed directly into the cell and participated within glycolysis. The hydrolysis of the disaccharides were dependent upon the production of enzymes, before participation in energy yielding pathways. The production of enzymes is an energy consuming process, which may have reduced the ability of the cell to accumulate zinc. It is also possible that during the course of the experiments the different industrial yeasts may have been at different stages of their growth cycle, if this was the case then the different uptake levels may be growth phase dependant (and influenced).

4.3.8 Zinc accumulation by yeast grown under energy limiting conditions

The ability of the industrial strains of *S. cerevisiae* (lager (Figure 4.3.8.1c), distillers (Figure 4.3.8.2c), wine (Figure 4.3.8.3c) and bakers (Figure 4.3.8.4c)) to sequester zinc when a metabolisable energy source was absent from the original growth medium was studied. This nutrient limiting condition had an obvious effect on the growth rates of the strains, with most of the yeast cells being restricted to approximately one doubling. This was probably due to the presence of storage carbohydrates (*e.g.* glycogen) in the seed culture cells. However, the effect of metabolisable energy source on the ability of these strains to sequester zinc was restricted to a biosorptive effect only, as there was no carbon source to power the membrane transporters to allow zinc to be taken up into the cell. Zinc accumulation increased over the time period examined. The maximal sequestration rate was observed with the lager yeast, in comparison to the 4 strains investigated. Zinc accumulation by lager yeast reached uptake levels of 565.36fg zinc/cell after the 24 hour examination period. The distillers yeast sequestered 177.6 fg zinc/cell, wine yeast 190.4 fg zinc/cell and the bakers yeast 283.7 fg zinc/cell. Due to the importance of the carbon source within the cells, the overall effect of this nutrient deprivation would have been detrimental to the cell, with the cell unable to generate energy for essential transport processes.

Figure 4.3.8.1: **Interactions between zinc ions and *S. cerevisiae* lager yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained no metabolisable energy source.

a)



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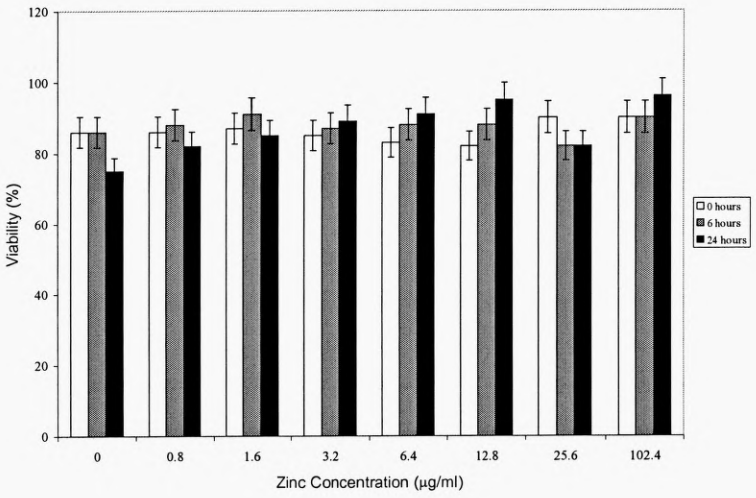

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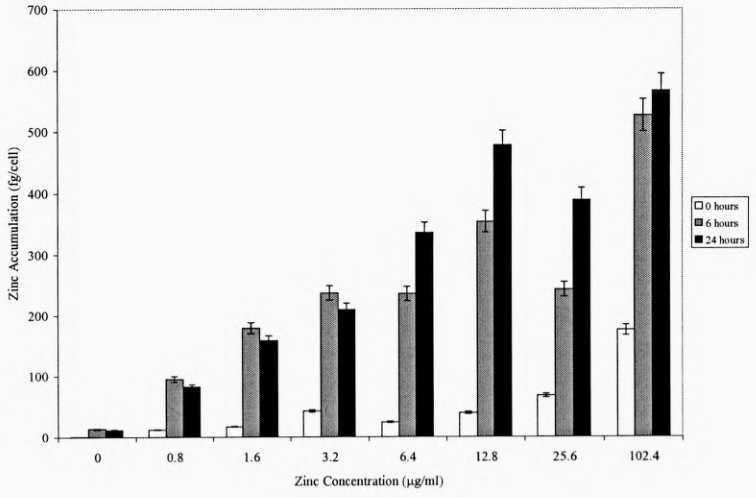
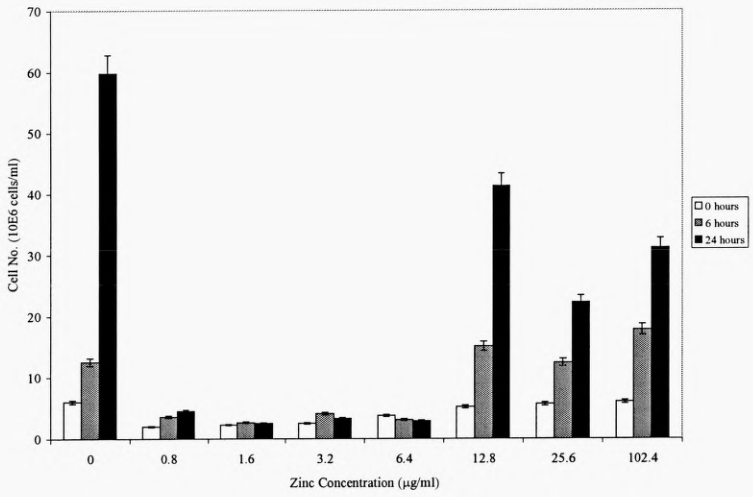


Figure 4.3.8.2: **Interactions between zinc ions and *S. cerevisiae* distillers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained no metabolisable energy source.

a)



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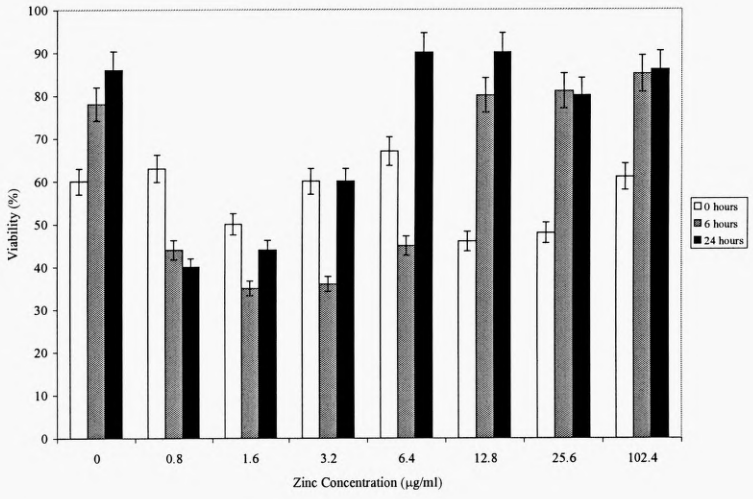

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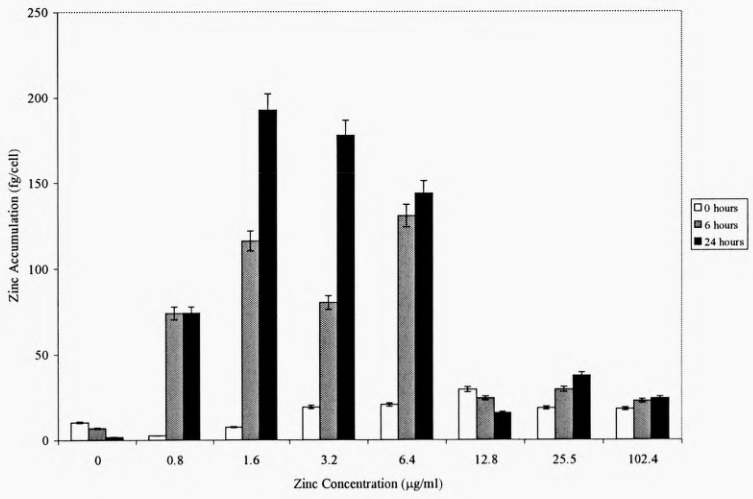
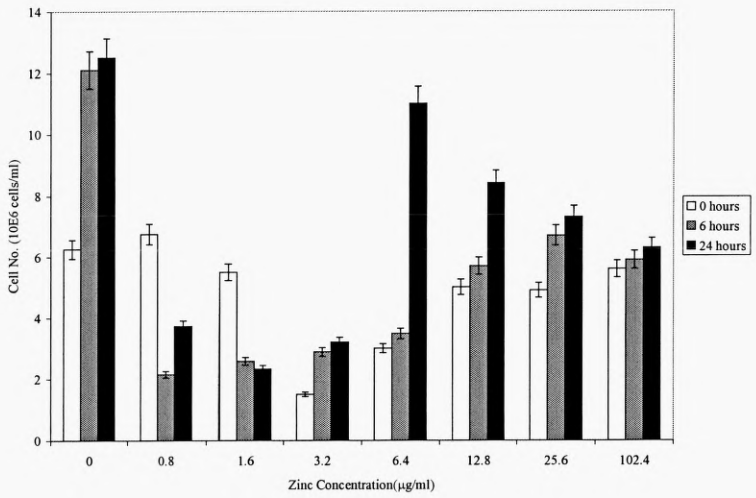


Figure 4.3.8.3: **Interactions between zinc ions and *S. cerevisiae* wine yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained no metabolisable energy source.

a)



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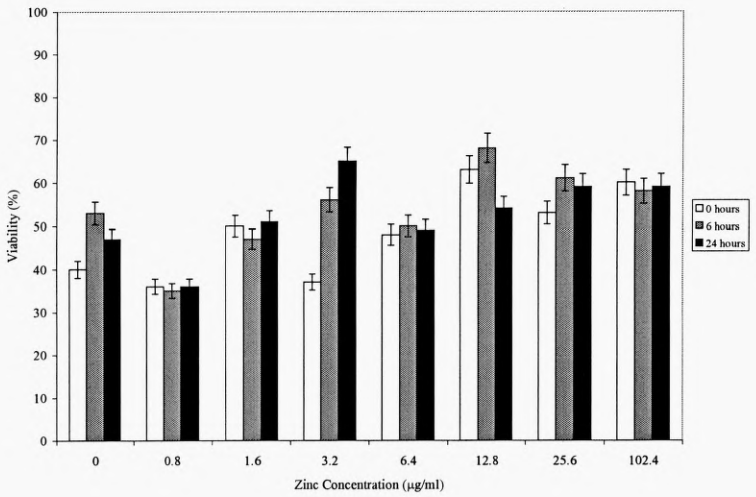

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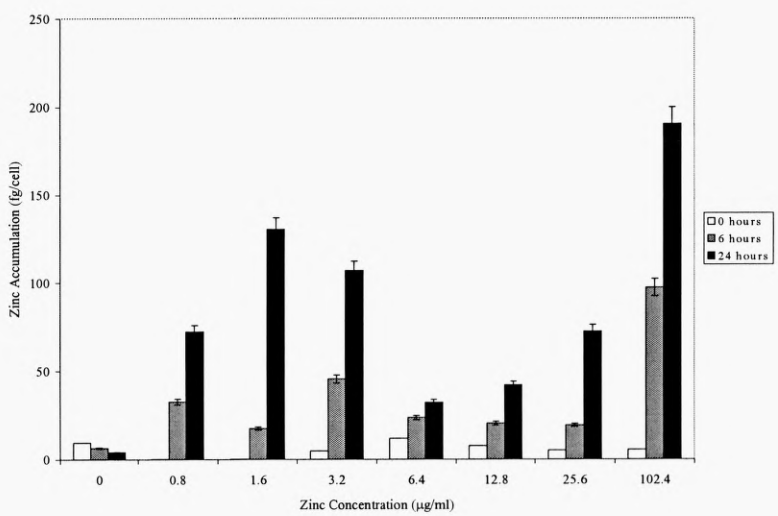
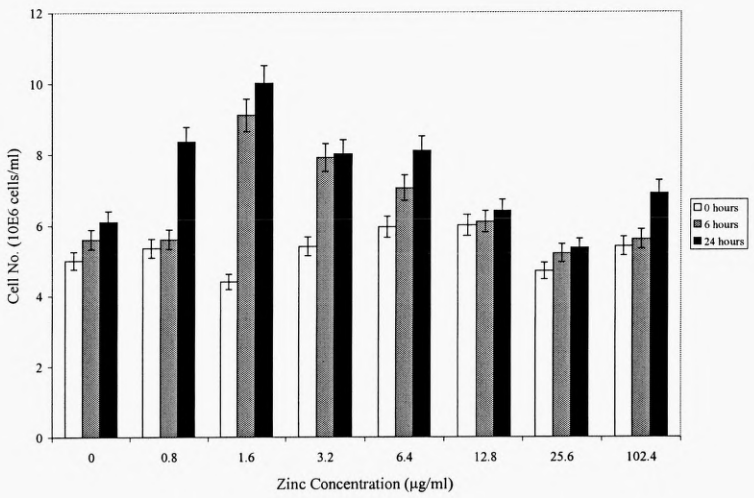


Figure 4.3.8.4: **Interactions between zinc ions and *S. cerevisiae* bakers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 25°C at pH4.5. This media contained no metabolisable energy source.

a)



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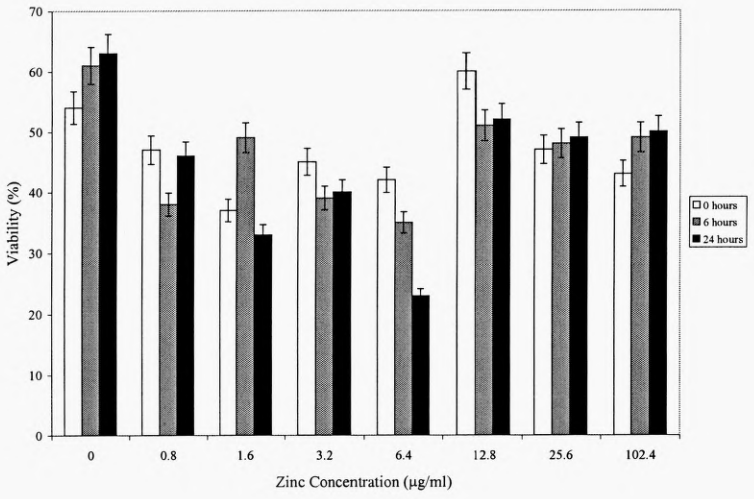

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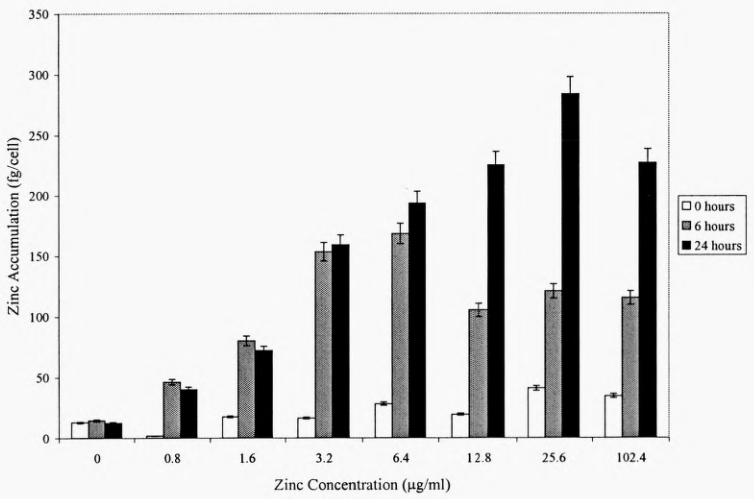


4.3.9 Influence of temperature on the ability of industrial yeasts to sequester zinc

The plasma membrane is very important for controlled accumulation of zinc ions by the industrial strains of *S. cerevisiae* examined. As mentioned in the Chapter 4 introduction, at low temperatures there are changes in the structure of this biological membrane. Also at low temperatures, the metabolic capability of the cell is markedly reduced and the rate at which chemical reactions occur is also affected. The uptake of zinc was also inhibited at 4°C. The ability of the yeast to sequester the ions intracellularly was reduced and the availability of the surface binding sites may also be affected in this low temperature. Figures 4.3.9.1-4.3.9.4 demonstrated the growth, viability and the zinc accumulation ability of the investigated industrial yeasts: lager, distillers, wine and bakers, respectively. It was at first apparent that cellular growth and reproduction was inhibited. However, viability of the lager yeast in zinc supplemented medium (Figure 4.3.9.1 b) remained relatively unaffected and this may be due to the protective effect of divalent cations in the stabilisation of the plasma membrane during periods of yeast stress (Walker, 1998b). Wine yeast sequestered the greatest amount of zinc initially (time 0) with 135 fg zinc/cell, the biosorptive effect of the other yeasts was 97.9 fg zinc/cell (lager yeast); 86.8 fg zinc/cell (distillers) and 77.8 fg zinc/cell (bakers yeast). The ability of industrial yeasts to sequester zinc when subjected to a temperature shock was still possible, however, the changes in the plasma membrane must be having a detrimental effect on the zinc transport proteins. This could have been demonstrated through the isolation and determination of plasma membrane proteins using SDS-PAGE. Zinc uptake by the studied industrial yeasts was still evident after 6 hours with the following uptake rates were achieved 69.7 fg zinc/cell (bakers yeast); 120 fg zinc/cell (distillers yeast); 124 fg zinc/cell (wine yeast) and 127 fg zinc/cell (lager yeast). The maximal level of zinc accumulated by the distillers yeast was 193fg zinc/cell after a 24 hours period, with the other yeasts sequestering 140 fg zinc/cell (lager yeast), 133 fg

zinc/cell (wine yeast) and 54 fg zinc/cell (bakers yeast). Over all the time intervals examined, bakers yeast sequestered the least amount of zinc, with no obvious pattern observed for the other species.

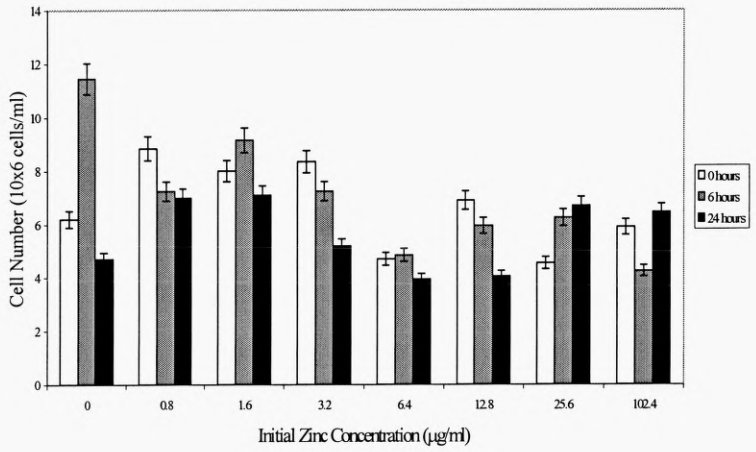
Figure 4.10: Summary of zinc accumulation by industrial strains of *S. cerevisiae* at a temperature of 4°C.

Yeast Strain	Maximum zinc accumulated over time (fg/cell)		
	0 hours	6 hours	24 hours
Lager yeast	97.9	127	140
Distillers yeast	86.8	120	193
Wine yeast	135	124	133
Bakers yeast	77.8	69.7	54

When this data is compared with results reported in Figures 4.3.4.1, 4.3.5.1, 4.3.6.1 and 4.3.7.1, it is clear that for the lager, distillers and the bakers yeast zinc uptake was reduced at this lower temperature. At this lower temperature the accumulation of zinc would probably be restricted to a biosorptive effect only, due to the changes in the plasma membrane structure and the possible derepression of the *ZRT* genes which control zinc accumulation in *S. cerevisiae*. This difference in the biosorptive effect might indicate a difference in availability of binding site within the mannoprotein section of the cell wall. This in turn might indicate that there may be slight intra-strain differences in the structure or in the abundance of the mannoprotein within the cell wall.

Figure 4.3.9.1: **Interactions between zinc ions and *S. cerevisiae* lager yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 4°C at pH4.5. This media contained glucose as the metabolisable energy source.

a)



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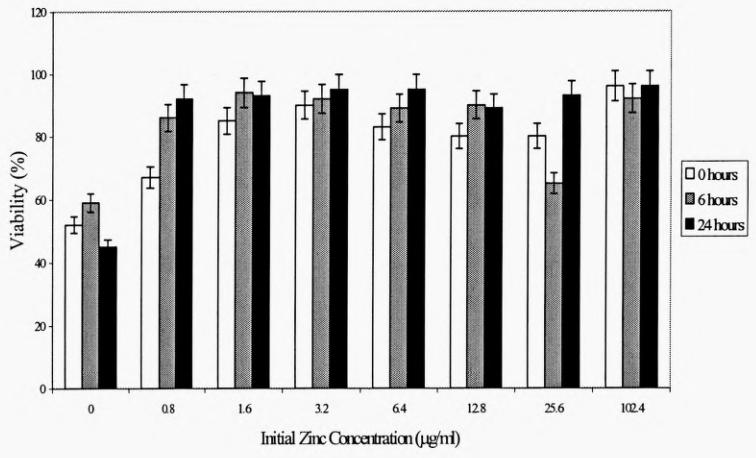

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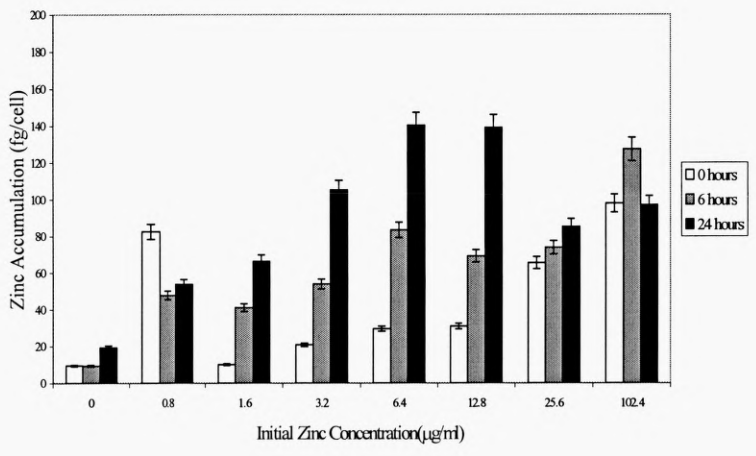
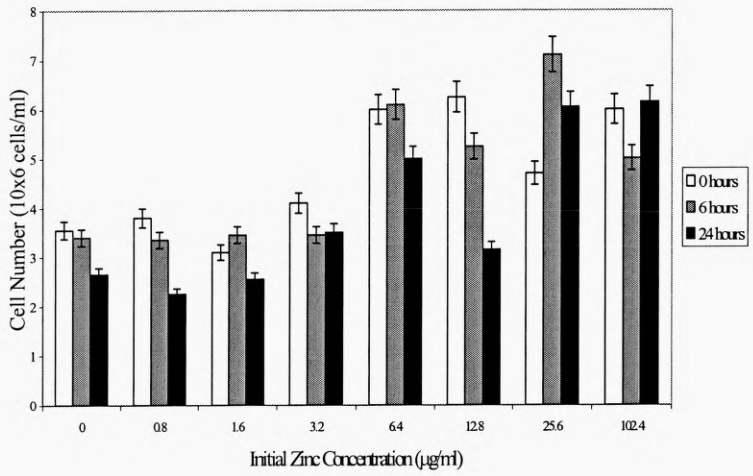


Figure 4.3.9.2: **Interactions between zinc ions and *S. cerevisiae* distillers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 4°C at pH4.5. This media contained glucose as the metabolisable energy source.

a)



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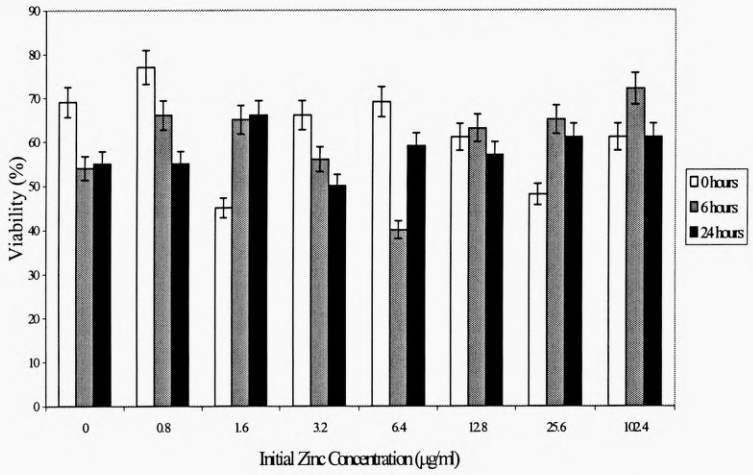

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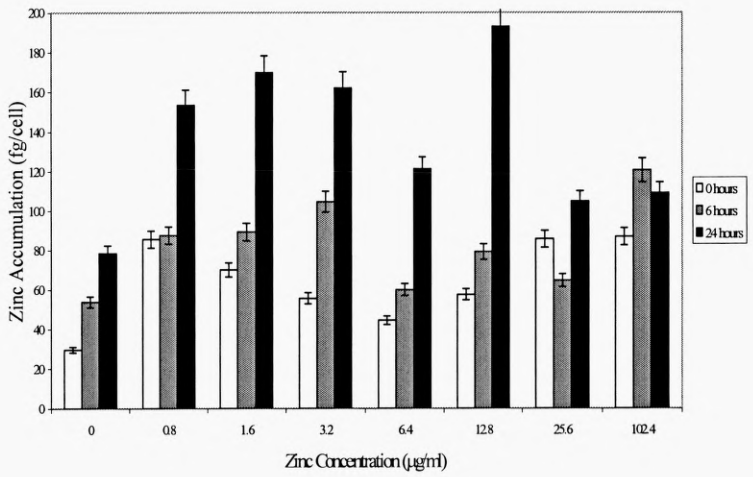
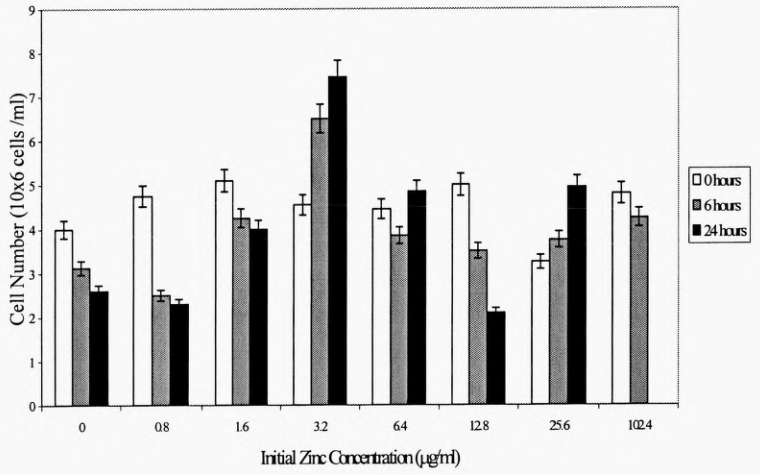


Figure 4.3.9.3: **Interactions between zinc ions and *S. cerevisiae* wine yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 4°C at pH4.5. This media contained glucose as the metabolisable energy source.

a)



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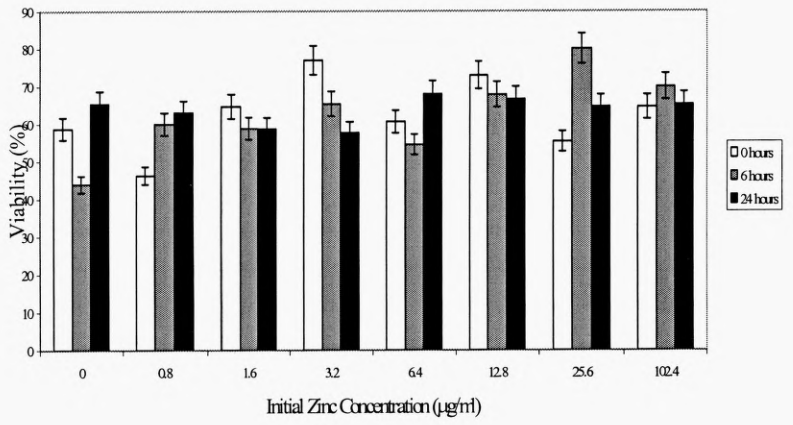

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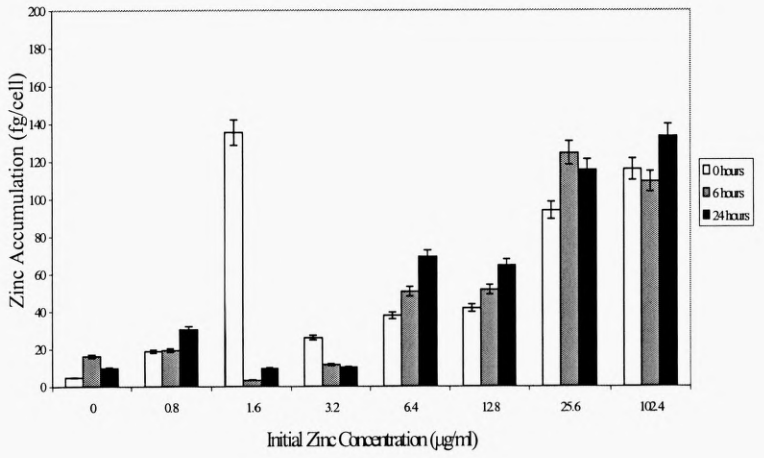
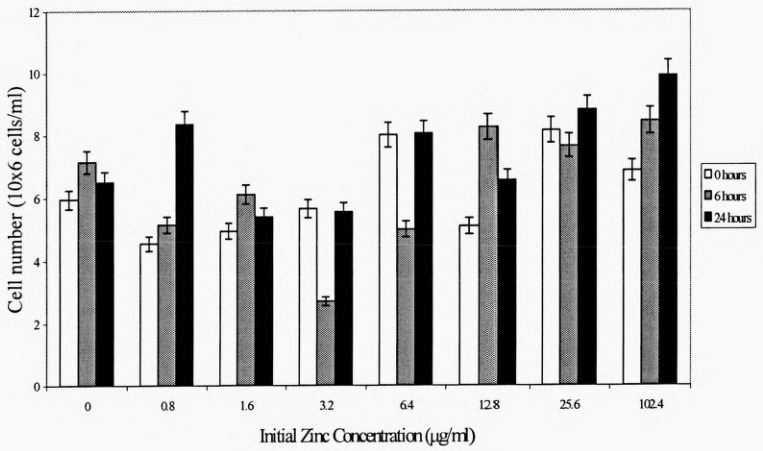


Figure 4.3.9.4: **Interactions between zinc ions and *S. cerevisiae* bakers yeast**. The growth (a), viability (b) and the zinc accumulating ability (c) was determined over a 24 hour period, in YPDM which contained various initial concentrations of zinc, at 4°C at pH4.5. This media contained glucose as the metabolisable energy source.

a)



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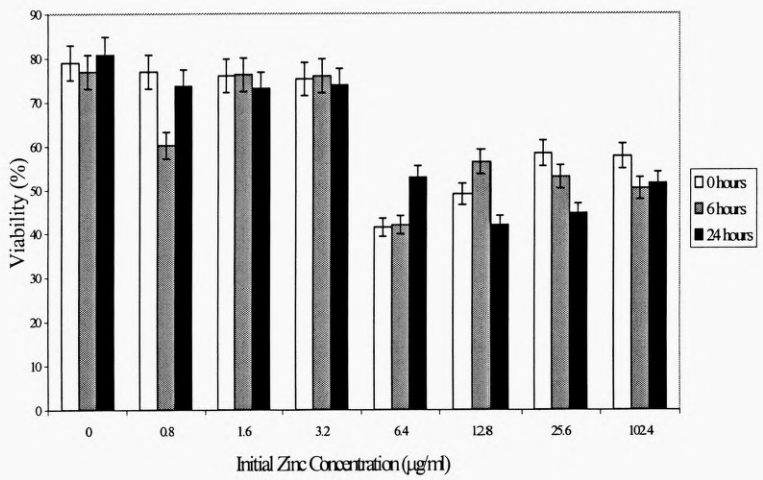

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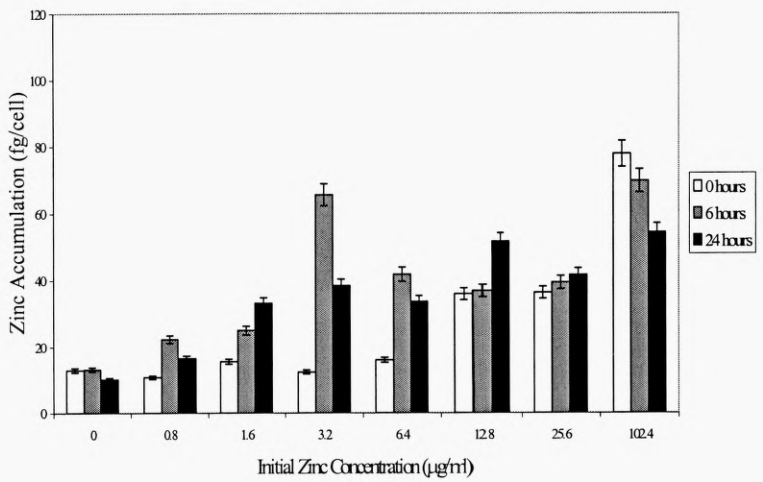


4.4 Conclusion

In conclusion, the uptake of zinc was appreciable in industrial strains of *S. cerevisiae,* with high levels of sequestration achievable. Accumulation levels were affected by the metabolisable energy source supplied, and by growth conditions including temperature and pH. Results from this Chapter indicated that for maximum growth only the lager strain required additional zinc, whereas the distillers, wine and bakers strains all achieved the greatest growth rates when the media was unsupplemented with zinc, indicating intra-species differences in the requirement and uptake of essential nutrients. The cells were not deficient in this important ion as the AAS analysis demonstrated that the cells that were grown in a media which was unsupplemented, that the cells still had some zinc present. When examining the effect of metabolisable energy source on zinc sequestration it would appear that over the time period examined that monosaccharides stimulated greater uptake in the wine and the bakers yeast. The disaccharides initially stimulated greater uptake in the lager yeast and the distillers yeast, however, after 24 hours the maximum uptake achieved with these 2 species was with the monosaccharides fructose and glucose, respectively. Therefore, after a period of 24 hours monosaccharides stimulated greater zinc accumulation than disaccharides, with maximal accumulation by the lager yeast grown in YPDM containing fructose. This may indicate that zinc uptake may be correlated to carbon source utilisation, therefore, energy generation. The utilisation of fructose within *S. cerevisiae* consumes one less ATP than the catabolism of glucose, and fructose stimulated greater levels of uptake than glucose. One way of determining if this was the case would be by measuring the intracellular ATP levels using the Firefly bioluminescence assay (Hysert and Morrison, 1977) and compare it to the zinc accumulation rates over the later time intervals. Another hypothesis that may explain the difference in the intracellular accumulation rates between the individual strains may be due to differences in the genes which govern zinc

accumulation. This may be further investigated by determining the mRNA levels or the protein expression levels for the strains using SDS-PAGE and blotting techniques, and determining if there was a difference between the strains.

The influence of temperature (4°C) on the ability of *S. cerevisiae* to sequester zinc may be due to the changes in the structure of the plasma membrane. This lower temperature will drive the cells to conserve energy, reducing the energy available to transport the zinc into the cells. At this low temperature genetic operations within the cell will have wound down, allowing only essential functions to continue operating. Therefore, in low temperatures the zinc transport genes may be depressed, as will the energy that drives the processes. This culmination of events at the level of the plasma membrane structural changes will undoubtedly affect the ability of the cell to accumulate all nutrients including zinc. Another consideration which might explain the differences in the uptake levels at 0 time, may be intra-strain differences in the cell wall structure with particular emphasis on the mannoprotein layer which is involved in the binding of the individual zinc ions. This difference may have a profound effect on the number of cysteine residues which are responsible for the binding of the individual zinc ions.

The utilisation of yeast biomass in bioremediation programmes is, therefore, possible with results demonstrating that high amounts of zinc can be accumulated. For maximal uptake, a temperature controlled process would be desirable, as temperature is highly influential in the amount of zinc sequestered by the cells. Viable biomass sequesters high amounts of zinc initially, with increased amount over time, and more zinc is accumulated in the presence of a monosaccharide based metabolisable energy source. The possibility of using relatively healthy waste biomass from the brewing sector would be a fast, cheap and favourable alternative to cleaning-up zinc containing waste effluents, as this yeast accumulated 23.06 μg/ml of zinc at time 0.

The knowledge obtained from these investigations may influence brewers to pre-condition yeasts at the propagation stage. This would allow for a high intracellular pool of zinc (and pre-conditioning may be possible with other divalent cations), which the yeast cells may utilise during industrial fermentations, as many industrial growth media (*e.g.* molasses) may be deficient in zinc.

During a typical lager fermentation, the process is conducted at 8°C. This low temperature may influence the zinc uptake ability of the yeast, and a zinc deficient yeast culture will not be functioning optimally. Therefore, the fermentation performance of yeast and efficiency of industrial production may be adversely affected.

Chapter 5

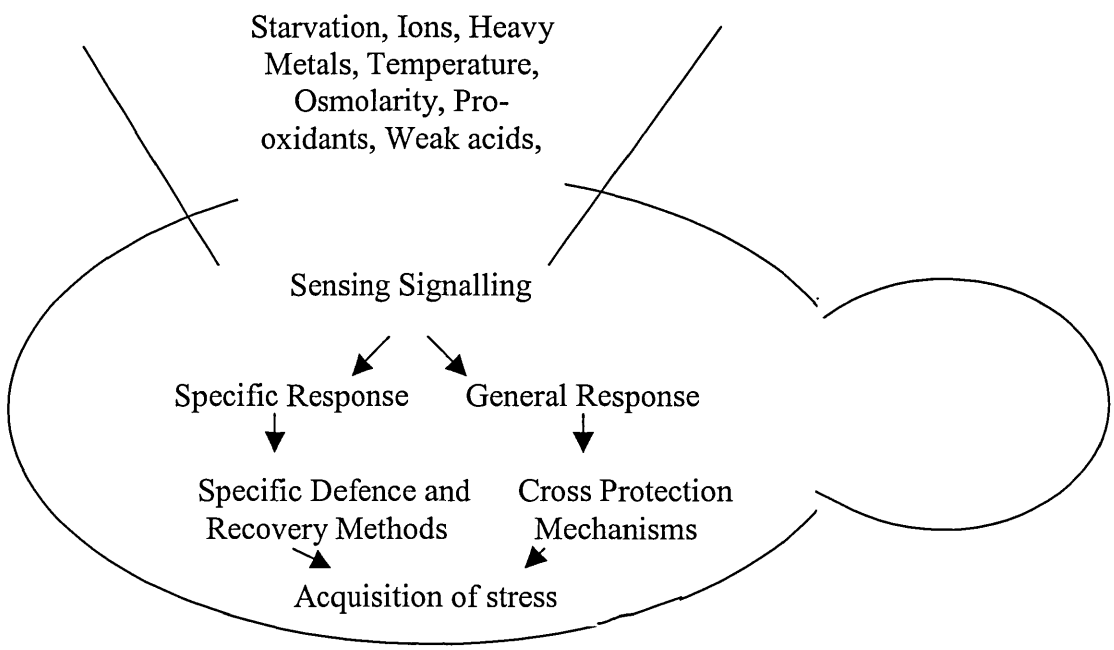
Relationship between yeast cell physiological state and zinc accumulation

5.1 Introduction

During the life cycle of a typical culture of *S. cerevisiae*, cells may experience several stressful situations. In industrial processes, such stresses may arise during: propagation, primary, secondary and post fermentation procedures (Walker, 1998b). In unicellular organisms, stress conditions may be broadly defined as environmental factors which result in a reduction in the growth rate of the cellular population. In industrial strains of *S. cerevisiae* these stresses may be due to fluctuations in temperature, low pH, presence of heavy metals, nutrient limitation, oxidative changes, CO_2 pressure and ethanol toxicity (Smart *et. al.*, 1995). These stresses may adversely affect the overall performance of the yeast cells and influence the ability of such cells to effectively utilise sugars and produce ethanol. Stressful situations do not occur as single events in the lifecycle of the cells, and the development of a protective mechanism against one stressful situation may confer a cross-protective mechanism across a range of situations. The protective mechanisms in yeast are illustrated by a rapid molecular response which may be either general or specific. However, both responses will result in the acquisition of stress tolerance (see Figure 5.1). Primarily when yeast cells encounter stressful situations there is an increase in the levels of specific proteins (*e.g.* heat shock proteins) and also in the levels of certain storage carbohydrates *i.e.* trehalose or glycogen. Adverse environmental conditions will also cause alterations in the membrane lipid composition (Fargher and Smith, 1995), ion exchange processes (Gadd, 1993), and in the case of oxidative stress when there is an increase in the production of free radicals the synthesis and activities of superoxide dismutase enzymes is

elevated (Birch, 1997). Figure 5.1 illustrates the actions of *S. cerevisiae* in response to stress situations.

Figure 5.1.1: Diagrammatic representation of stresses which affect a yeast cell demonstrating the intracellular stress responses.



(adapted from Siderius and Mager, 1997)

Through the activation of signalling pathways, this results in an increase in the expression of a wide variety of genes (Siderius and Mager, 1997), which collectively confer stress-protection within the cell.

Concerning temperature stresses, *S. cerevisiae* will grow through a range of temperatures, but a shift to a temperature exceeding 40°C (generally regarded as sub-lethal) will result in an increase in the degree of saturation within the fatty acid component of the plasma membrane. This will, in turn, result in the membrane becoming more fluid. *S. cerevisiae* cells with an increase in the degree of saturation are thought to be more heat tolerant (Swan and Watson, 1997). Swan and Watson (1997) have also reported that ethanol tolerance may be correlated with a high level of membrane fluidity in both *S. cerevisiae* and *Kloeckera apiculata*. This increase in the level of saturated lipids in the membrane, is the opposite of when the cells are subjected to a low temperature shock, where the plasma membrane takes on a more arranged structure. This response to elevated temperature (and ethanol) will, therefore, render the membrane more permeable to ions and low molecular weight metabolites (Walker, 1998b).

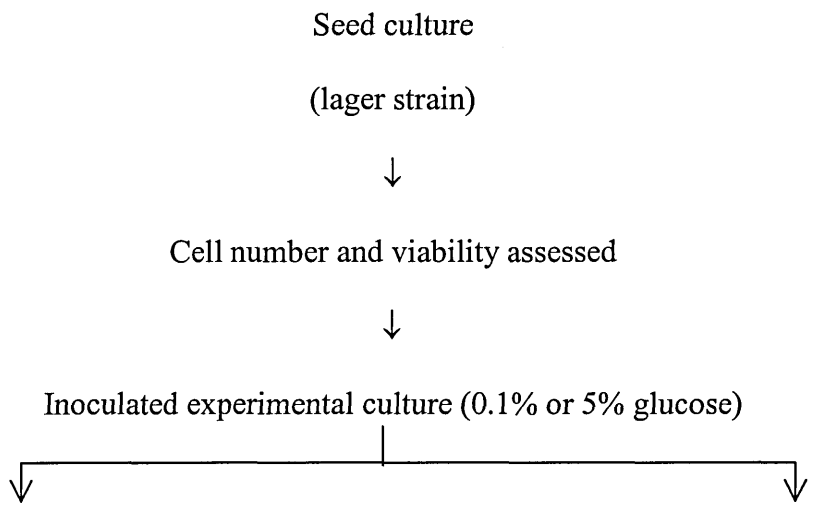
The aims of this chapter were to specifically:

- Examine how the metabolic "state" of the cell can influence the zinc accumulating ability of a lager brewing strain

- Determine if zinc can alleviate the effects of a chemical and a physical stress on lager yeast

5.2 Experimental approach

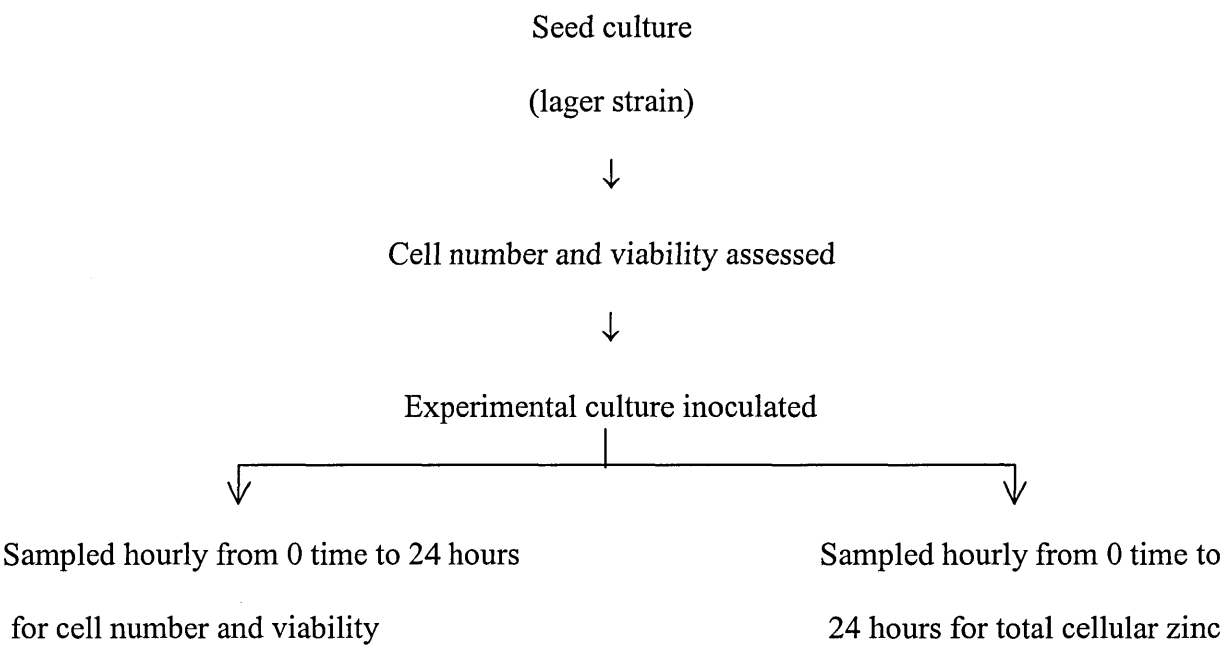
5.2.1 Studies on the effect of yeast metabolic state (fermentation vs. respiration) on growth, viability and zinc accumulation



Using this approach, it was possible to influence the metabolic state of the cell and to determine if the metabolic state of the lager yeast could influence the amount of zinc sequestered.

5.2.2 Studies on zinc accumulation during yeast cell growth

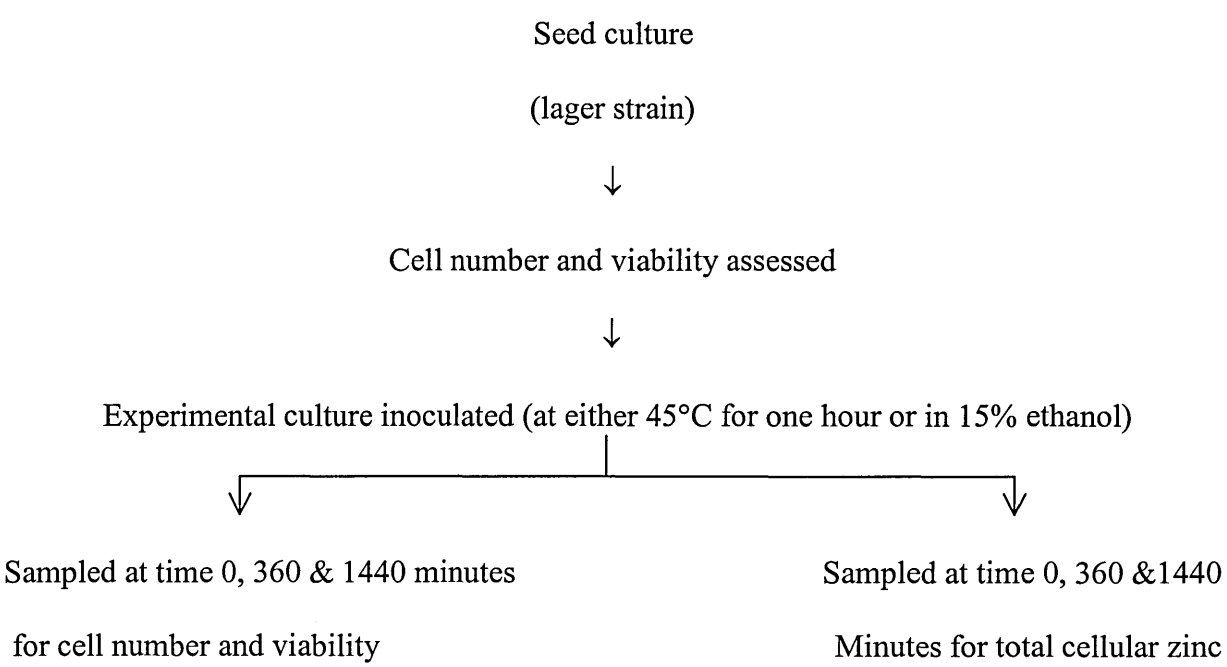
The following diagram demonstrates the experimental procedure adopted in order to determine the growth cycle of *S. cerevisiae* lager yeast.



Using this approach it was possible to determine the growth curve of a lager brewing strain, grown in YPDM, containing 3% glucose as the metabolisable energy source, and supplemented with 0.8μg/ml zinc.

5.2.3 Studies on the effect of zinc on temperature and ethanol stressed cells

The following diagram shows the experimental approach adopted to determine the effect of zinc on cells which were subjected to chemical and physical abuse (this methods was adapted from Walker *et. al.* 1996).



5.3 Results and Discussion

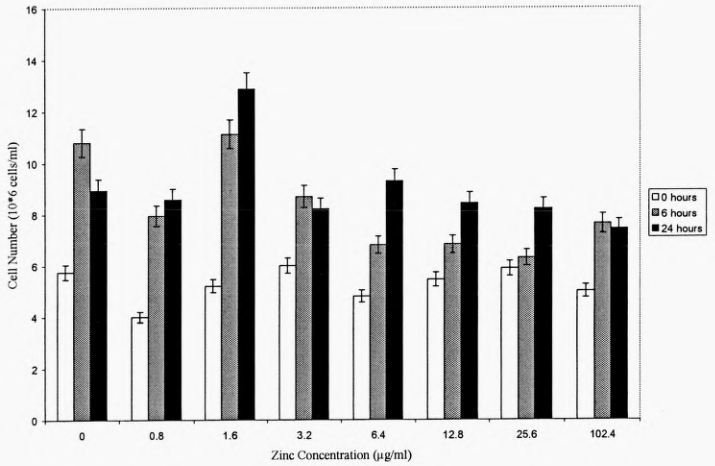
The ability of lager yeast to grow and accumulate zinc when metabolising in a respiratory mode (0.1% glucose) was investigated. It was found that, growth appeared to halt after approximately one doubling. The respiring cells were grown in 0.1% glucose, and this allowed for the maximal cell density of $12.86x10^6$ cells/ml, when the initial zinc concentration was 1.6μg/ml (Figure 5.3.1a). The culture viability was generally unaffected by growth at this level (Figure 5.3.1b). The zinc accumulating ability of these cells increased over time and also increased over the concentration gradient *i.e.* the more zinc in the media the greater the level of accumulation, when the data was expressed as zinc uptake per population (μg zinc/ml culture). When the data was represented on a per cell basis (Figure 5.3.1c), there was increased uptake over the initial time intervals of 0 and 6 hours. This indicated that the cells were actively accumulating zinc, suggesting a cellular demand for zinc during the early stages of growth.

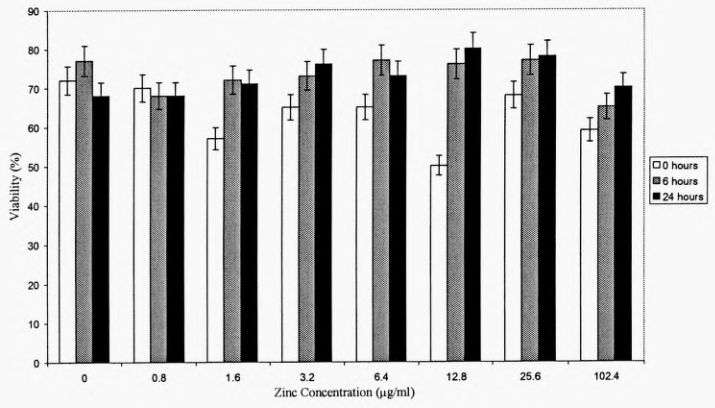
When studying the growth of lager yeast cells when grown in defined media supplemented with 5% glucose, it was observed that the cells followed a fermentative metabolic route. This was evident by the production of the fermentative metabolite ethanol (data not represented). The cell number was greatly influenced by this additional glucose, with growth peaking at $69.0x10^6$ cells/ml (Figure 5.3.2a). The viability of the inoculated cells was initially poor (Figure 5.3.2b), but viability recovered over the time period of the experiment. The cells were examined for their ability to sequester zinc and at a glucose concentration of 5% were seen to increase zinc uptake over the time period studied. Zinc accumulation on a per cell basis (Figure 5.3.2c) increased until 6 hours, but, once the cells entered the logarithmic period of growth, the zinc was diluted into the daughter cells. Therefore, it appears that there is actually less zinc accumulated, suggesting that zinc was not constantly accumulated, as the majority of the zinc was seen to be sequestered over the

initial time period of 0 time with a slight increase over the remaining times (6hours and 24 hours). The level of zinc accumulated was 1.814μg/ml after 24 hours, from an available concentration of 12.8μg/ml, when the cells were respiring. This equated to 215fg zinc/cell. However, fermenting cells at the initial concentration of 12.8μg/ml sequestered 1.964μg/ml, an increase of 0.15μg/ml, which is equivalent to an increase of 7.64% over the respiring cells. The maximum zinc accumulated by the fermenting cells after 24 hours was 2.229μg/ml, which was achieved from the initial concentration of 102.4μg/ml. This was an increase of 0.415μg/ml, or 18.6%, from when the cells were respiring. This shows that fermenting cells have a greater cellular demand for zinc than respiring cells. The increased glucose concentration stimulated greater zinc uptake into the fermenting lager yeast. The stimulation of zinc uptake by glucose is apparent through the comparison of lager yeast grown under such contrasting initial sugar concentrations, suggesting that the facilitated diffusion of glucose into the lager yeast stimulated the cells to actively uptake zinc ions from the growth media. The increased uptake of zinc into fermenting cells has also been reported by Stelik-Thomas *et. al.* (1997), where *S. cerevisiae* grown under anaerobic conditions accumulated 62% more zinc than cells grown under aerobic or semiaerobic conditions, again demonstrating that fermenting cells have a greater need for enhanced levels of intracellular zinc. Therefore, these enhanced levels of intracellular zinc may be utilised by fermentation associated enzymes *i.e.* alcohol dehydrogenase.

This pattern of divalent cation uptake was also demonstrated with magnesium. Walker and Maynard (1997) demonstrated that media bioavailability, cellular uptake and subsequent metabolic utilisation of magnesium ions by yeast cells appear to be pre-requisites for the achievement of maximum fermentation activity and that ethanol production and glucose consumption were highly dependent on the availability of Mg ions in the growth medium.

Figure 5.3.1: Zinc accumulation in *S. cerevisiae* lager yeast encouraged to grow in a respiratory mode of growth. These cells were supplied with YPDM containing 0.1% glucose, at a temperature of 25°C, on a shaking platform set at 200rpm.





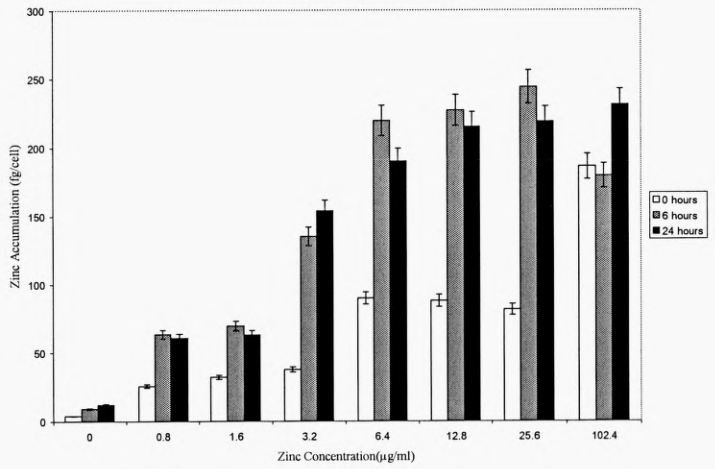


Figure 5.3.2: Zinc accumulation in fermenting cells of *S. cerevisiae* lager yeast. The cells were cultured in the YPDM containing 5% glucose as the metabolisable energy source, at 25°C.

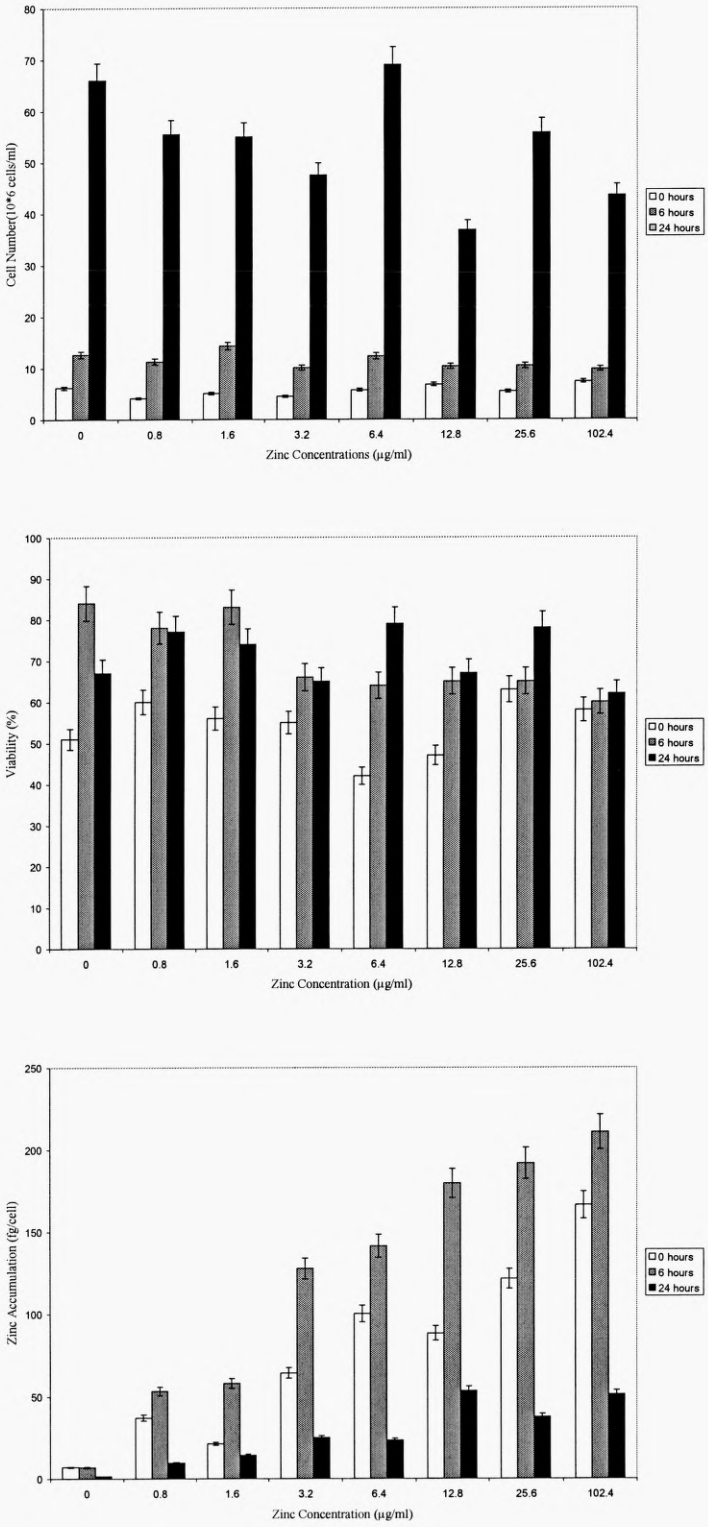


Figure 5.3.3: Growth curve of *S. cerevisiae* lager yeast grown in defined media containing 3% glucose and 0.8μg/ml of zinc.

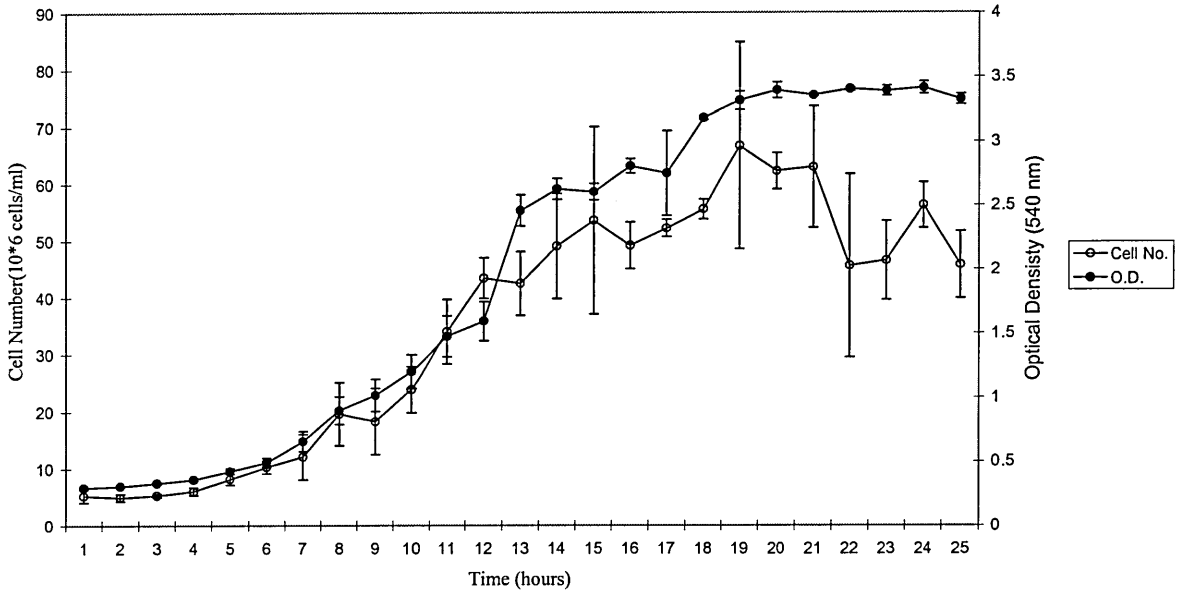
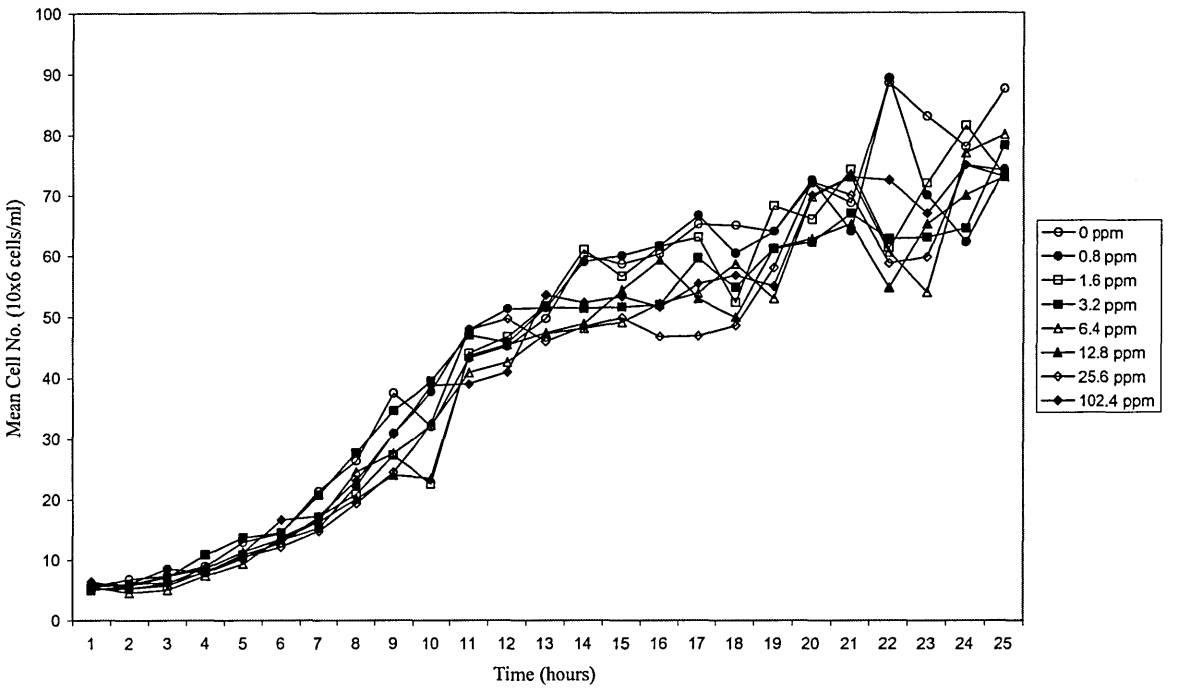


Figure 5.3.4: Growth and viability of a lager brewing yeast over a 24 hour time period. Cells were cultured in YPDM, containing 3% glucose as the metabolisable energy source, and various initial concentrations of zinc. Standard deviations were calculated on the individual data points, but were not presented on these graphs for clarity.

a)



b)

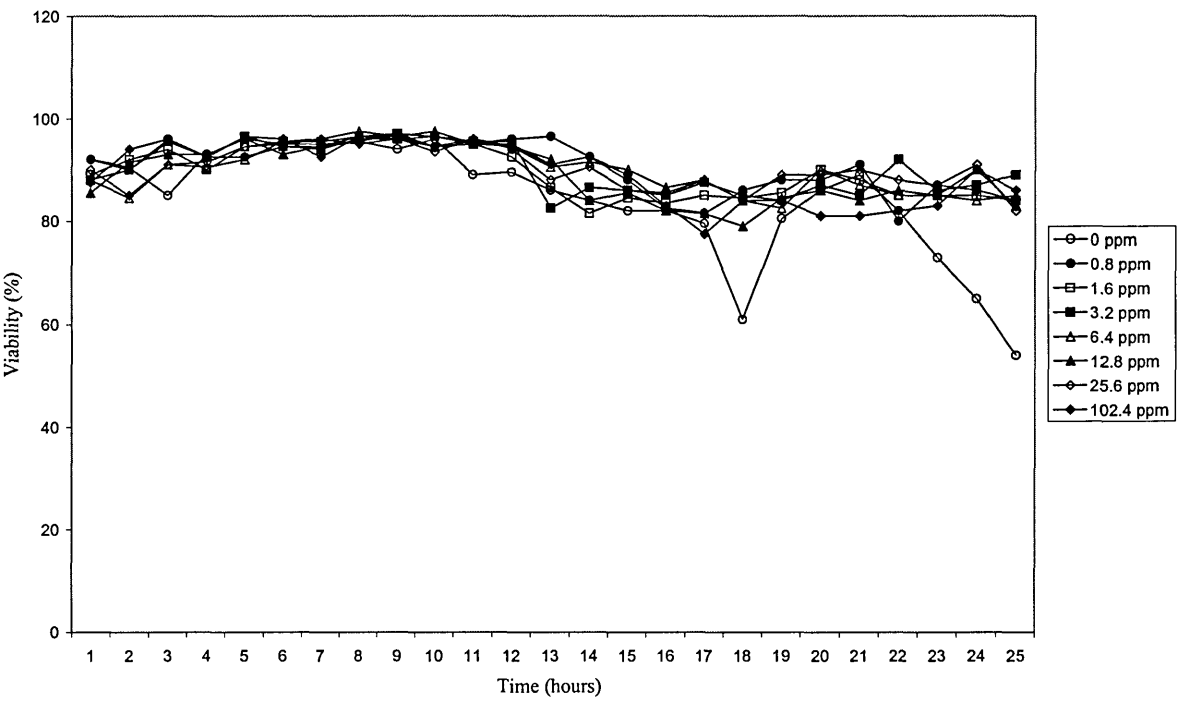


Figure 5.3.5: Zinc accumulation in lager brewing yeast cultured in YPDM containing 3% glucose as the metabolisable energy source, containing various initial concentrations of zinc, over a 24 hour time period. Standard deviations were calculated on the individual data points, but were not presented on these graphs for clarity.

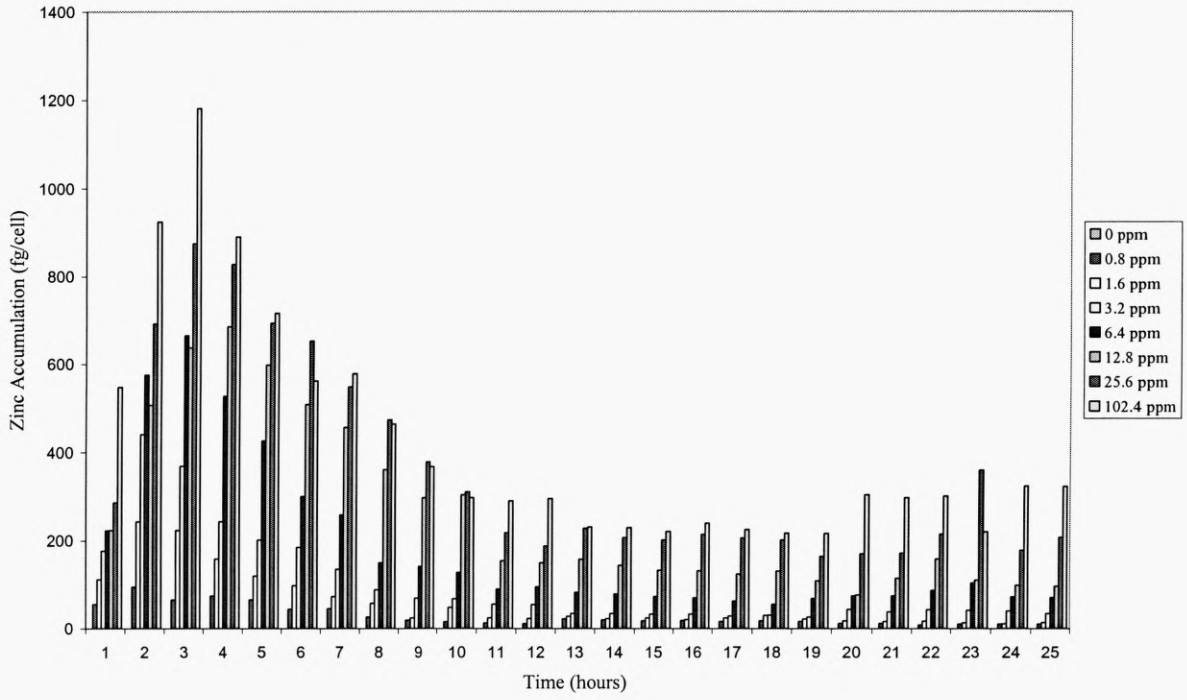


Figure 5.3.3 demonstrated *S. cerevisiae*, when grown in a defined media which contains 3% glucose as the metabolisable energy source, conformed to the traditional growth curve, which passed through lag, exponential and stationary phases. When the lager yeast was studied with respect to its growth pattern under various zinc concentrations, ranging from 0-102.4μg/ml (Figure 5.3.4a), all of the cultures followed the proposed pattern of growth (*i.e.* the same growth pattern as Figure 5.3.3). On studying the specific growth rates (μ) of the individual cultures, (Figure 5.3.6) it would appear that the maximal growth of the lager yeast was stimulated by 6.4 μg zinc/ml. Reports that zinc limitation inhibits the growth of *S. cerevisiae*, causing bud inhibition and cell cycle arrest in the G1 phase of the cell cycle (Stewart and Russell, 1998) was not obvious (see Figure 5.3.6), as the culture grown in zinc unsupplemented media had a growth rate of 0.0218 h^{-1}, as this was not the lowest growth rate observed.

Figure 5.3.6: Table comparing the specific growth rates (μ) of *S. cerevisiae* lager yeast when grown in a minimal media containing various initial concentrations of zinc.

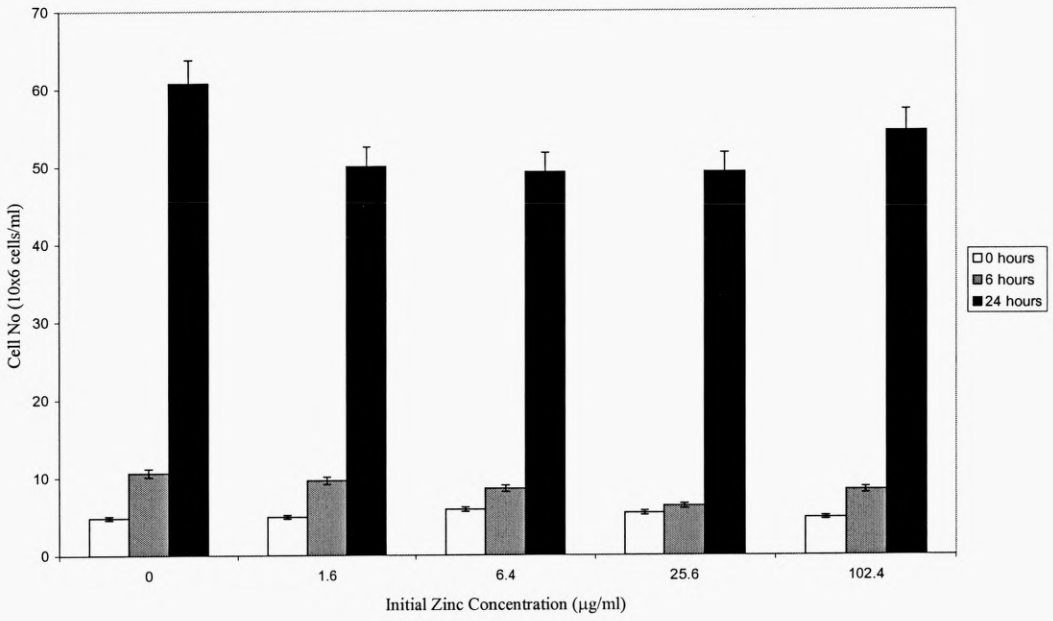
Initial Zinc Concentration (μg/ml)	0	0.8	1.6	3.2	6.4	12.8	25.6	102.4
Specific Growth Rate (μ) h^{-1}	0.0218	0.0277	0.0267	0.019	0.041	0.031	0.0176	0.234

However, on examination of cell viability (Figure 5.3.4b), when there was no supplemented zinc in the experimental media the viability began to decline at approximately 20 hours. Cultures that were supplemented with zinc remained constantly and highly viable.

The accumulation of zinc by a growing culture (Figure 5.3.5) appeared to peak during the lag and the early exponential phase at approximately 1200 fg zinc/cell. Zinc accumulation increased again during the early stationary phase (beginning at 20 hours). Stehlik-Thomas *et. al.* (1997) have also examined the effect of zinc on alcoholic fermentations and have reported that zinc accumulation in *S. cerevisiae* occurred during the first 8 hours during a batch fermentation process. This data (as well as the data in figure 5.3.5) is in accordance with the cyclic accumulation of zinc by *Candida utilis*, with uptake also occurring during the lag and the early exponential phases of growth (Faillia and Weinberg, 1977). This cyclic accumulation of zinc in the yeasts *S. cerevisiae* and *C. utilis* appears to follow similar patterns of accumulation, with uptake optimal during the first 8-9 hours of growth. This may demonstrate an enhanced requirement for zinc during the early stages of growth, when the cells are more affected by stressful situations. It is conceivable that this represents a "conscious" accumulation of zinc during this stage of growth to alleviate the symptoms of stressful situations by acting as a membrane stabilising agent. During normal growth and development zinc is influential as a functional and structural support in many enzyme systems that are essential for various biochemical and physiological functions. It is, therefore, logical that zinc should be sequestered during the early stages of growth.

Figure 5.3.7: The effect of zinc on *S. cerevisiae* cells, which have been subjected to a 1 hour 45°C heat shock, the varying time periods follow the growth and the viability of the cells over 24 hours, with 0 time representing the pre-heat shock condition.

a)



b)

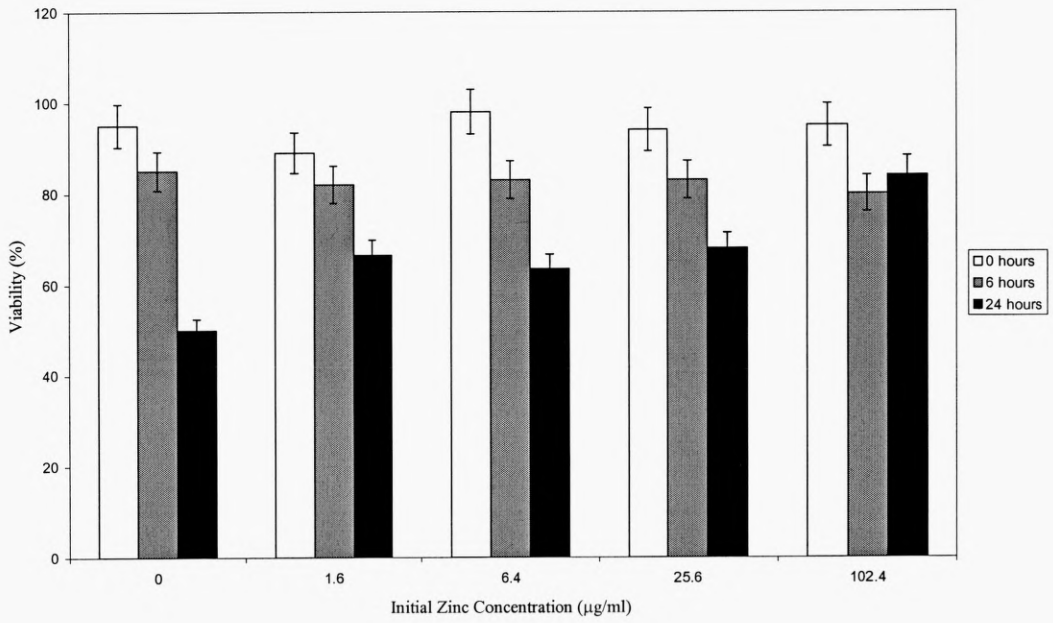
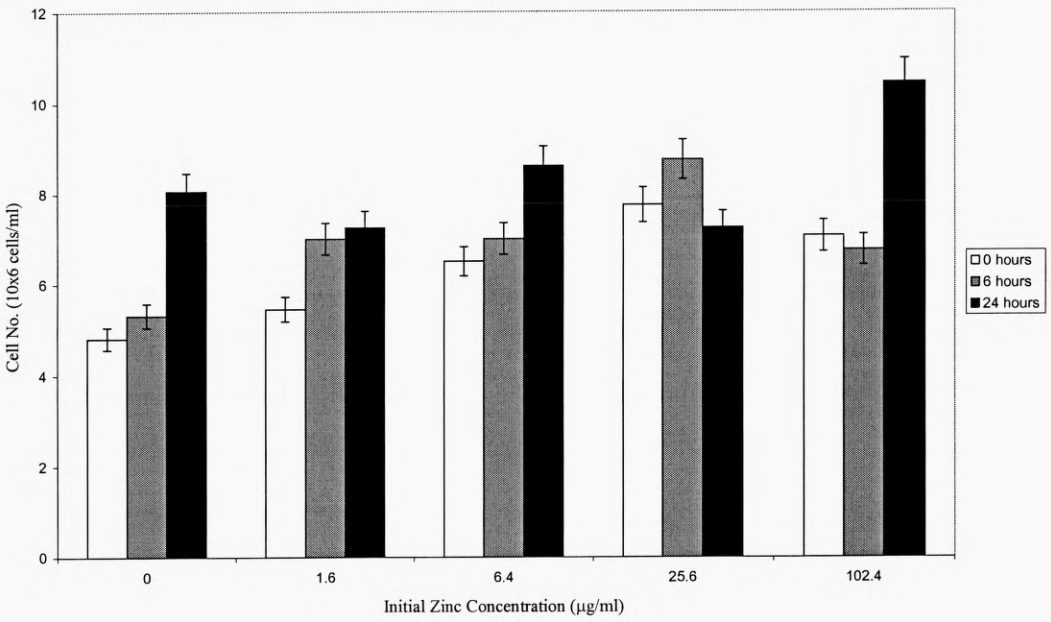
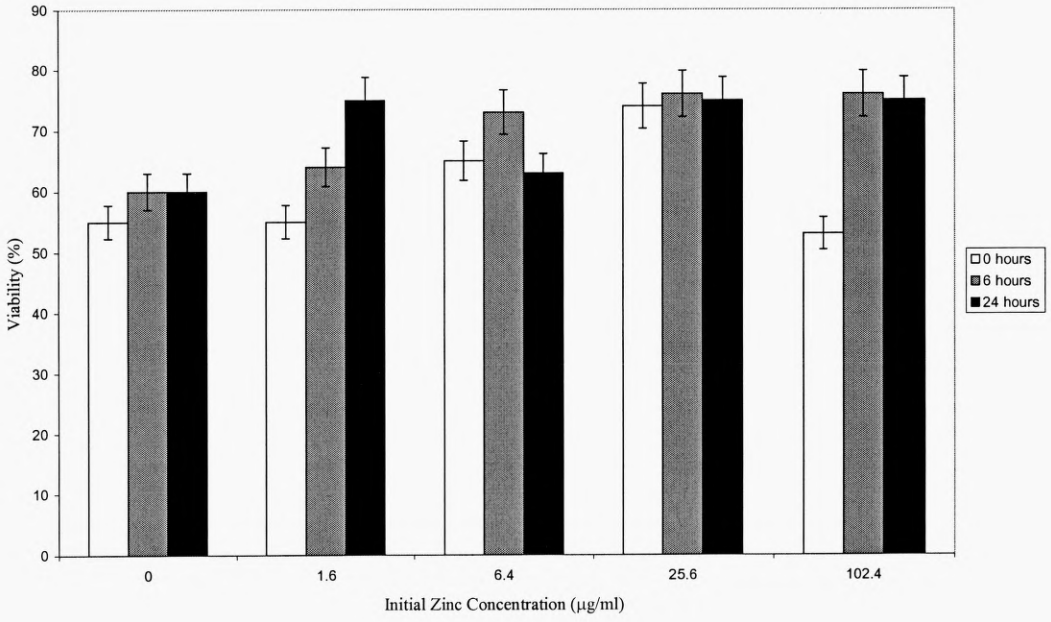


Figure 5.3.8: The effect of zinc on *S. cerevisiae* cells, which have been exposed to a 15% ethanol shock, over a 24 hour period. The 0 time period represents pre-ethanol shock conditions.

a)



b)



The cells which were subjected to heat shock at 45°C for 1hour did not appear to have been inhibited with respect to the growth rate (Figure 5.3.7a). However, the culture viability (Figure 5.3.7b) was reduced, with a significant reduction in the viability of the unsupplemented zinc culture. The culture which was supplemented with a high level of zinc (102.4 μg/ml) appeared to be the least affected by the heat shock conditions, over the time period examined. During the heat shock experiment, the stabilisation properties of zinc may have been exploited by the yeast cells plasma membrane protecting the cell from the injurious effects of temperature stress. In all of the cultures that were supplemented with zinc, the viability remained higher than the unsupplemented culture. Therefore, the divalent cation zinc may have a role with respect to the derepression of heat shock proteins synthesis.

The ability of yeasts to tolerate the toxic effects of ethanol is of paramount importance, due to the commercial production of ethanol by industrial strains of *S. cerevisiae*. The ability of these cells to grow and remain viable when subjected to a 15% ethanol shock was examined (Figures 5.3.8 a, b). The first obvious effect of the ethanol on the cells, was that growth was remarkably reduced. Ethanol tolerance in *S. cerevisiae* is greater than in other organisms (Swan and Watson, 1997). However, the plasma membrane of *S. cerevisiae* is thought to be the prime target for ethanol toxicity. The cultures were supplied with 3% glucose, but glucose accumulation is non-competitively inhibited in the presence of ethanol (Cartwright *et.al.* 1986). Therefore, the growth rate may have been inhibited due to the lack of glucose and other essential nutrients accumulated. On studying the viability of lager yeast subjected to a 15% ethanol shock, the unsupplemented cells appeared to have a lower viability than the cells that were supplemented with zinc (Figure 5.3.8b). This pattern was similar to the pattern observed with the heat shock experiment. Therefore, zinc appeared to confer a

protective effect on lager yeast under the stressful situations examined as this was illustrated in the improved viability results of supplemented cultures.

The stress of heat shock (a temperature greater than 37°C) and ethanol concentration (above 4-6%v/v) evoke similar responses in the yeast *S. cerevisiae*. These stresses cause similar changes in certain plasma membrane proteins, for example, by reducing the levels of plasma membrane H^+-ATPase (Piper, 1995). In addition, the heat shock protein 104 (Hsp104), will contribute a general protective mechanism against temperature and ethanol abuse (Piper, 1995). However, the production of stress proteins does not appear to have an irreversible effect on the viability of the cultures studied.

Walker (1998b) has reported on the protective effect of magnesium under similar heat shock and ethanol shock conditions. Such findings indicate that magnesium supplemented media has a degree of protection on lager, wine and distillers yeast viability when the cells were subjected to ethanol and heat stress. From the current data, it appears that zinc plays a similar role. This suggests that certain divalent cations may have a general role in the protection of the yeast cell from the stressful effects of fermentations. If metal ions do confer the observed general protective effect, the possibility of pre-conditioning yeasts with particular ions might prove a worthwhile exercise for the brewer.

5.4 Conclusion

In conclusion, zinc uptake by *S. cerevisiae* was stimulated by glucose, with fermentative cells displaying a greater level of uptake than respiring cells. Cells which were fermenting accumulated more zinc over the same time period as when the cells were respiring. This may suggest that fermenting cells had a greater requirement for zinc. Zinc is necessary for alcohol dehydrogenase production and activity, as well as the stimulation of protein synthesis. When the cells were metabolising fermentatively zinc was essential for DNA synthesis and stabilisation.

This Chapter also demonstrated the cyclic accumulation of zinc uptake during the yeast growth cycle, with maximum uptake achieved in lag and early logarithmic phase cells. The accumulation of zinc during these stages aswell as influencing the production of many enzyme families, may convey a protective effect to cells that were affected by stressful situations. For example, ethanol concentrations above 6% v/v are generally quite toxic to yeast cells. The majority of ethanol is produced during cellular budding, and so, an increase in zinc uptake during this time may be able to prevent the adverse effects of the ethanol on the plasma membrane, either, by acting to stabilise the membrane or possibly by producing general and specific stress proteins.

The results of Walker (1998b) demonstrated similar effects with magnesium. It is therefore possible that the observed protective response to zinc may be a general effect of divalent cations.

Chapter 6

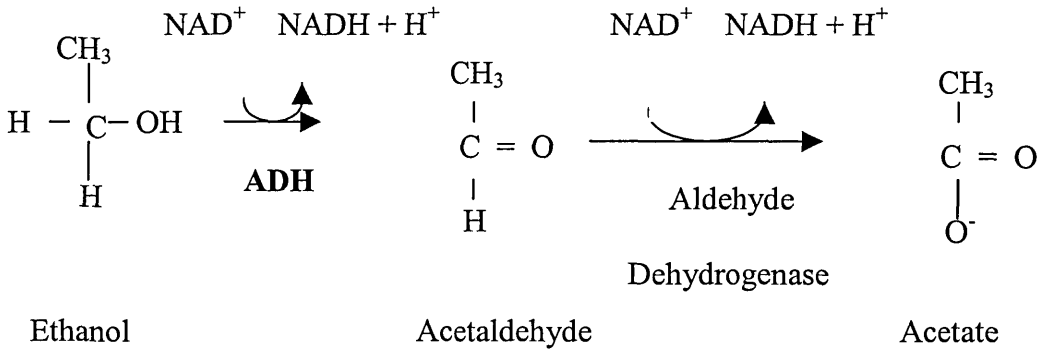
Relationship between cellular zinc and yeast physiology

6.1 Introduction

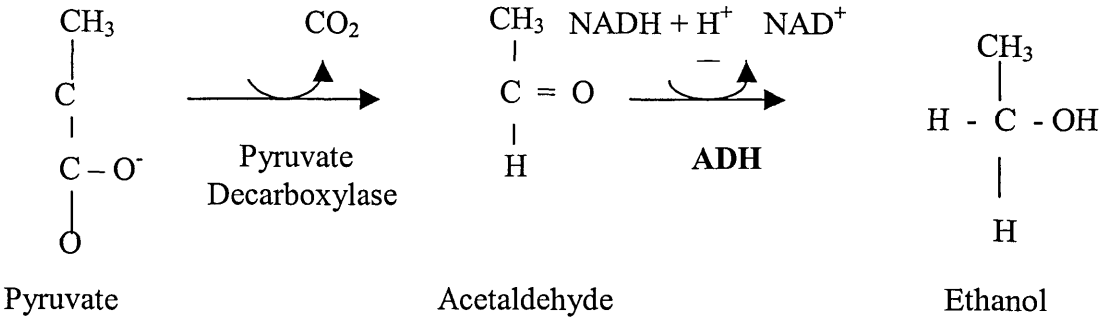
All biochemical reactions that occur within a cell are catalysed by enzymes. Alcohol dehydrogenases (ADH) are soluble enzymes that are relatively non-specific and can use several basic alcohols as substrates. ADH's catalyse the interconversion of simple alcohols and aldehydes *i.e.* ethanol can be oxidised to form acetaldehyde and the reverse is possible through the reduction of acetaldehyde. These enzymes are not unique to yeast cells and are produced in higher eukaryotes including humans. Their function, however, may be different depending on the organism in question. In higher eukaryotes, including animals and humans the main function of ethanol dehydrogenase is to oxidise any ethanol build up, therefore providing a mechanism for detoxification (Voet and Voet, 1990). Figure 6.1.1 summaries the reduction and the oxidation processes which involve ADH. This oxidation reaction will convert ethanol to acetaldehyde, then acetic acid, and finally into acetyl Co-A which can feed directly into the Citric acid cycle. In yeasts, the reaction proceeds in the reductive direction, which provides a route directly to fermentative metabolism. This pathway is entered through the pyruvate dehydrogenase bypass, as the pyruvate is decarboxylated by pyruvate decarboxylase to acetaldehyde, which is in turn reduced to produce ethanol (Walker, 1998a). In this way, the cell is able to maintain a redox balance since NAD reduced in glycolysis is re-oxidised by the terminal ADH in fermentation.

Figure 6.1.1: Diagram demonstrating the function of alcohol dehydrogenase in humans and in yeast.

In Humans



In Yeast



Yeast alcohol dehydrogenases (1 and 2) are tetrameric structures, which are located in the cytosol (Heick *et. al.* 1969). These enzymes have a molecular weight of approximately 150kDa and the component subunits are approximately 36kDa (Kägi and Vallee, 1960). Each sub-unit incorporates 2 atoms of zinc, with the respective atoms acting in a catalytic and a conformational capacity (Magonet *et. al.* 1992; De Bolle *et. al.*, 1997). In horse liver alcohol dehydrogenase (HLADH), one zinc atom is found in the catalytic site of the enzyme, and is located bound to 2 cysteine and 1 histidine residues (Cys-46, His-67 and Cys-174). Although the crystal structure for yeast alcohol dehydrogenase (YADH) has not been published, it is speculated to be similar to HLADH (Magonet *et. al.* 1992). The second zinc atom in HLADH is known to be associated with 4 cysteine residues (97, 100, 103 and 111), which have been found to exist in nearly all ADHs including YADH (Magonet *et. al.* 1992). Speculative evidence reports that a second zinc atom may also be located at this site and acts as a structural support (Magonet *et.al.* 1992). Other divalent cations are known to stabilise YADH1 and these include the chloride salts of calcium and magnesium (De Bolle *et. al.* 1997). It has been demonstrated that these divalent cations participate in an inter-subunit binding, which in turn stabilises the reduced form of ADH1 (reduced ADH1 is the form located *in vivo*) (De Bolle *et. al.*, 1997)

In yeast, the zinc atom, which is complexed within a cysteine-histidine-cysteine ligand, appears to destabilise the aldehyde group, and the resultant release of a hydride ion from NADH reduces the substrate to ethanol.
(http.www.udel.edu/chem/bahnson/epstein/mechanisms.html).
The necessity of zinc for the production of yeast alcohol dehydrogenase is, therefore, essential.

This chapter specifically aimed,

- To determine the effect of zinc on the specific activities of yeast alcohol dehydrogenase.

- To determine the effect of zinc on the overall ethanol output in response to carbon source.

6.2 Experimental approach

The yeast cultures that were employed in this Chapter were the industrial strains of *S. cerevisiae* introduced in Chapter 4. The yeast strains examined with respect to the enzyme assays was the lager brewing strain. Yeasts were grown in YPDM, described in chapter2. The yeasts were grown up in seed cultures and then repitched into fresh media at approximately $5x10^6$ cells/ml.

Determination of total protein was necessary in order to quantify the specific activities of alcohol dehydrogenase produced by lager yeast over time, and depending on the initial zinc concentration. A slight deviation from the method stated in Chapter 2, instead of digesting the cells with alkali, the total protein was determined on ruptured whole cells, the procedure for which is described in the preparation of samples for the alcohol dehydrogenase assay (Chapter 2).

The experimental approach adopted allowed for the determination of ADH activity in lager brewing yeast. The yeast was grown in YPDM, which was supplemented with varying concentrations of zinc and the effect of zinc on the ADH activity was examined using the protocol in Chapter 2. In order to investigate the ethanol yield of the zinc cultured cells, gas chromatography was used (Chapter 2).

6.3 Results and Discussion

6.3.1 Determination of ADH activity within *S. cerevisiae* lager yeast

The determination of alcohol dehydrogenase in a homogenised yeast cell can be assayed by taking advantage of the fact that nicotinamide adenine dinucleotide (NAD) is the co-factor. At a wavelength of 340nm, NAD displays weak absorbance while NADH absorbs significantly. The rate of an ADH catalysed reaction can, therefore, be determined by mixing the enzyme with a suitable alcohol and the co-factor NAD, and following the conversion of NAD to NADH. This conversion is displayed by an increase in absorbancy values at 340nm over time. The rate of the reaction is expressed as a change in the absorbance per unit time (ΔA/time). To calculate the amount of enzyme produced by *S. cerevisiae* lager yeast, which was grown in a defined media which contained different initial concentrations of zinc, the Beer-Lambert equation was employed. The Beer-Lambert equation, which describes a relationship between the amount of light absorbed by a substance at a specific wavelength allowed the determination of the amount of alcohol dehydrogenase produced by the lager yeast, is represented below.

$\Delta A = E.C.L$ where; E = molar extinction co-efficient

C = concentration

L = path length (cm)

E, may also be referred to as molar absorptivity, which is a constant, and for NADH

$E = 6.22 \times 10^3\ dm^3\ cm^{-1} mol^{-1}$ (Reed *et. al.* 1998)

The spectrophotometer and cuvettes used gave a path length of 1cm.

This equation can, therefore, be rearranged to give:

$$C = \frac{\Delta A}{E.L}$$

ΔA was calculated by determining the gradient of the slope, M.

$$\Delta A = \frac{Y_2 - Y_1}{X_2 - X_1} = M$$

where, the y values (1 and 2) were the absorbance values, and the x values (1 and 2) were the corresponding time values at which the absorbancies were taken.

$$e.g.\quad M = \frac{0.203 - 0.132}{180 - 120\ (secs)} = \frac{0.071}{60} = 0.001$$

Therefore, to determine the actual amount of NAD which was converted to NADH, the following equation is an example:

$$C = \frac{\Delta A}{E.L} = \frac{0.001}{(6.22 \times 10^3).1} = \frac{0.001}{6.22 \times 10^3} = 0.00016 mM$$

The C is equal to the amount of NADH produced in moles. This value can therefore be used in order to establish the activity of ADH by the lager yeast. Figures 6.3.1 and 6.3.2 demonstrate the amount of the enzyme produced over time and the influence of zinc on the overall specific activity of ADH.

Figure 6.3.1: Influence of varying initial concentrations of zinc on ADH specific activity in *S. cerevisiae* lager yeast, at 0 hours. The enzyme activity is expressed as μmol NADH/mg protein/min.

Initial Zinc Conc. (μg/ml)	ΔA (min)	NADH Conc. (μM)	Total protein (mg/ml)	Enzyme Activity (μmol NADH/mg protein/min)
0	0.001	0.16	0.053	3.02
0.8	0.0009	0.145	0.024	6.04
1.6	0.0006	0.096	0.018	5.3
3.2	0.0011	0.177	0.024	7.38
12.8	0.0008	0.13	0.017	7.65
25.6	0.0006	0.0965	0.013	7.42
102.4	0.0007	0.1125	0.041	2.74

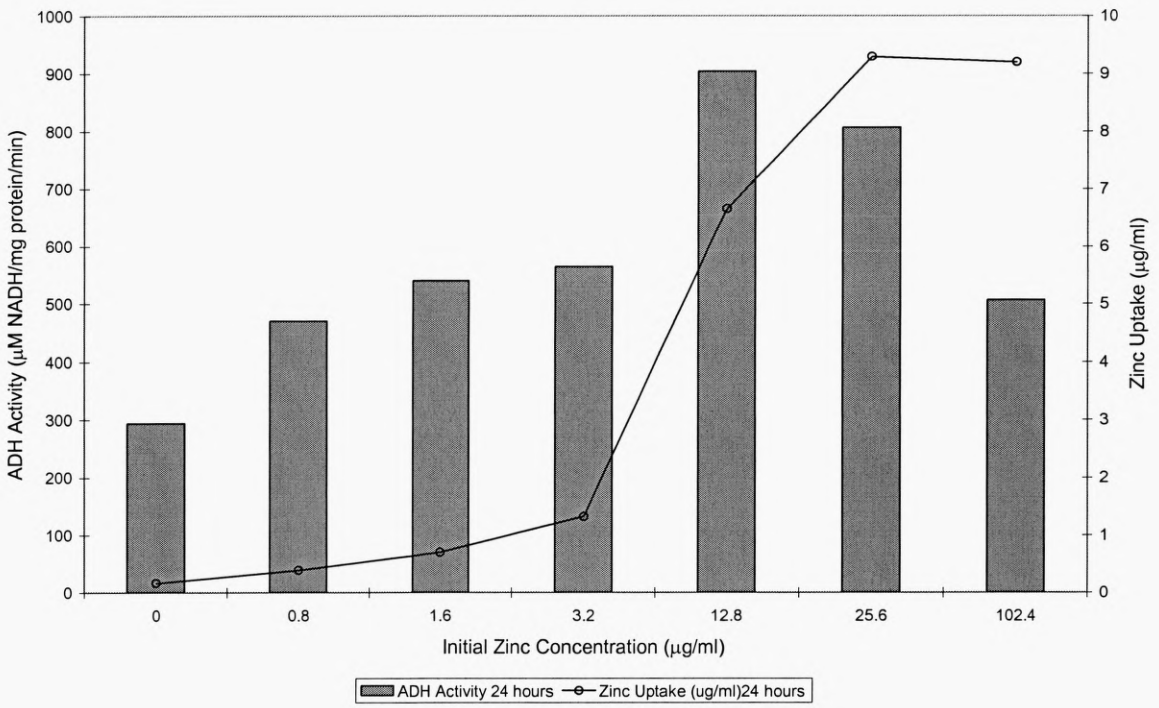
Figure 6.3.2: Influence of varying initial concentrations of zinc on ADH specific activity in *S. cerevisiae* lager yeast, at 24 hours. The enzyme activity is expressed as μmol NADH/mg protein/min.

Initial Zinc Conc. (μg/ml)	ΔA (min)	NADH Conc. (μM)	Total protein (mg/ml)	Enzyme Activity (μmol NADH/mg protein/min)
0	0.0003	48.2	0.164	293.9
0.8	0.0006	96.5	0.205	470.7
1.6	0.0005	80.4	0.149	539.6
3.2	0.0006	96.5	0.171	564.3
12.8	0.001	160	0.177	904
25.6	0.0008	129	0.16	806.25
102.4	0.0005	80	0.158	506.3

Figures 6.3.1 and 6.3.2, shows that zinc has a positive influence on ADH activities after 24 hours (in comparison to the yeast cells that were grown in unsupplemented zinc media). The enzyme activity generally increased during the time course, over the increasing in zinc concentrations, up to the level of 12.8μg/ml. This zinc concentration allowed for ADH activity to peak at 904 units, which is an increase of 610units when the media was unsupplemented with zinc, and an increase of 544 units from the standard commercial preparation- 360 units (Sigma Chemicals). Figure 6.3.1 and 2, show there is a general relationship between ADH activity and zinc accumulation. Generally, increased zinc uptake resulted in an increase in ADH activity. With a proposed 2 atoms of zinc necessary per sub-unit of ADH enzyme, enzyme production increased favourably up-to an initial zinc concentration of 12.8 μg/ml. The zinc concentrations greater than this did not stimulate as much enzyme activity suggesting that 12.8 μg/ml zinc was optimal for the production, stimulation and activity of this essential enzyme.

Experiments conducted by Heick *et. al.*(1969) demonstrated that bakers yeast grown in yeast nitrogen base containing 1.5% glucose, for 11 hours, then ethanol for 11 hours, resulted in alcohol dehydrogenase activity of 308 units. This yeast was not supplemented with respect to the mineral content, but the zinc content of YNB was 400μg/l. This is considerably lower amount of zinc than the zinc concentrations supplied in the YPDM. In comparison, *S. cerevisiae* grown in a defined media supplemented with varying amount of zinc resulted in a greater stimulation than the work of Heick *et.al.* (1969).

Figure 6.3.3: Correlation of zinc uptake and ADH activity in *S. cerevisiae* lager yeast cells grown in YPDM containing various initial concentrations of zinc and 3% glucose as the metabolisable energy source.



Although the relationship between ADH activity and zinc accumulation was general (increased ADH activity over the zinc concentration gradient up to an initial zinc concentration of 12.8 μg zinc/ml), the following question arose. Does this increase in enzyme activity in lager yeast influence the ethanol output? This was investigated and results shown in Figure 6.3.4.

Figure 6.3.4: Relationship between ADH activity and ethanol production in *S. cerevisiae* lager yeast after 24 hours, grown in YPDM containing varying initial concentrations of zinc and 3% glucose as the metabolisable energy source.

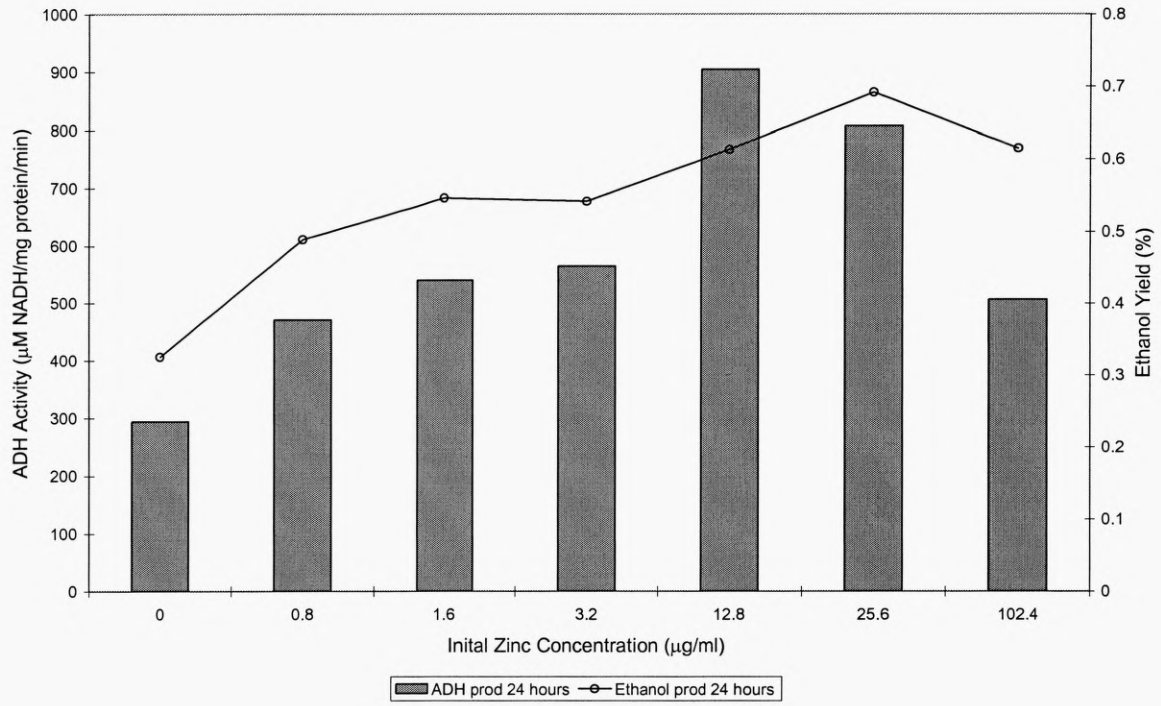


Figure 6.3.4 shows a close correlation, with increased alcohol dehydrogenase activity, with increased ethanol yield, over the 24 hour period studied. The increase in this metabolite production under the conditions studied demonstrated the stimulating effect of the divalent cation zinc on yeast fermentative metabolism. Possibly if the sugar concentration had been greater than 3%, there might have been a greater difference in the ethanol yield. The theoretical ethanol yield would be from 1g. glucose, 0.51g ethanol and 0.49g CO_2, however in practice a small amount of the glucose is used for biomass production, therefore the actual ethanol yields would be slightly less (Stewart and Russell, 1998).

Due to the influence of zinc on specific enzyme production and its subsequent positive influence on the ethanol yield, the next set of experiments were designed to examine the influence of both initial zinc concentration and metabolisable energy source on the ability of the 4 industrial relevant strains of *S. cerevisiae* to produce ethanol (3 strains exploited for their alcohol producing ability and a bakers strain).

6.3.2 Influence of zinc concentration on fermentation of different sugars in industrial strains of *S. cerevisiae*

The exploitation of industrial strains of *S. cerevisiae* is not restricted to ethanol production. However, this industry creates revenues of billions of dollars per annum. It is, therefore, essential that the metabolism and physiology of these yeasts are fully understood for maximum exploitation and commercial gain. Alcohol producing yeasts convert starting materials to suitable end products. In the case of lager yeast, which are strains of *S. cerevisiae*, fermentation performance has been described as "the ability of the yeasts to constantly metabolise wort constituents into ethanol and other fermentation products in order to produce beer with satisfactory quality and stability" (Russell and Stewart, 1995). The natural substrate for lager and whisky fermentations is malt wort, which consists of glucose, fructose, sucrose, maltose, maltotriose, and dextrins. Of these carbohydrates maltose is the predominant fermentable sugar present in wort.

For distillers yeasts, Figure 6.3.5.1 demonstrates the possible carbohydrates fermented and the respective end products.

Figure 6.3.5.1: Carbohydrate sources utilised by Distillers yeast (adapted from Walker, 1998a)

Raw Material	Fermentable Carbohydrate	Product
Malted Barley	Hydrolysed starch	Scotch malt whisky
Malt plus wheat or maize	Hydrolysed starch	Scotch grain whisky and neutral spirits
Malt plus rye	Hydrolysed starch	Bourbon whiskey
Grapes	Grape juice sugars	Brandy, Cognac, Armagnac
Fermented (spent) grape must/ grape residues	Grape juice sugars	Grappa
Sugar cane or beet molasses	Sucrose	Rum, neutral spirits
Potatoes, cereals plus malt	Hydrolysed starch	Vodka
Rice plus *Aspergillus oryzae*	Hydrolysed starch	Shochu
Palm oil	Various sugars	Raki
Agave tequilana	Hydrolysed inulin	Tequila
Cheese whey	Lactose	Neutral spirits, cream liqueurs

Many different yeasts are involved in wine making (Walker, 1999). Interestingly, *S. cerevisiae* may be thought of as a useful contaminant in the process (Mortimer and Polsinelli, 1999), as this yeast may not be present on grape skins and possibly arises from the winery fermentation areas (Deak, 1998).

Bakers yeast, which is utilised in the leavening of maltose rich dough, used to be obtained from the brewer in the form of "spent" yeast and it was usual to find the 2 industries on the same premises. Nowadays the production of bakers yeast has found its own industrial niche and is no longer living in the shadow of the brewer.

Mochaba *et. al.* (1996) have shown that zinc is actively taken up from the wort before fermentation begins and zinc accumulation is generally complete during the first 12 hours of cultivation (Stelik-Tomas *et. al.*, 1997). These studies are in agreement with the results from the previous chapters, which determined that zinc uptake was a biphasic response, occurring almost instantaneously. Due to zinc being incorporated into specific glycolytic

enzymes, it is logical that zinc uptake begins before the initiation of important metabolic pathways.

These industrial yeasts, therefore, all have intrinsic abilities to ferment various sugars sources supplied (glucose, fructose, maltose and sucrose). The following experiments were designed to study the influence of zinc on the fermentation of these sugars.

6.3.2.1 Zinc uptake and glucose fermentation by yeast

The effect of zinc on the ability of industrial strains of *S. cerevisiae* to ferment glucose was studied (Figure 6.3.6a-d). The cell number, viability and zinc uptake ability of different yeasts was reported in Chapter 4 (The effect of physical and chemical parameters on zinc uptake by industrial yeasts).

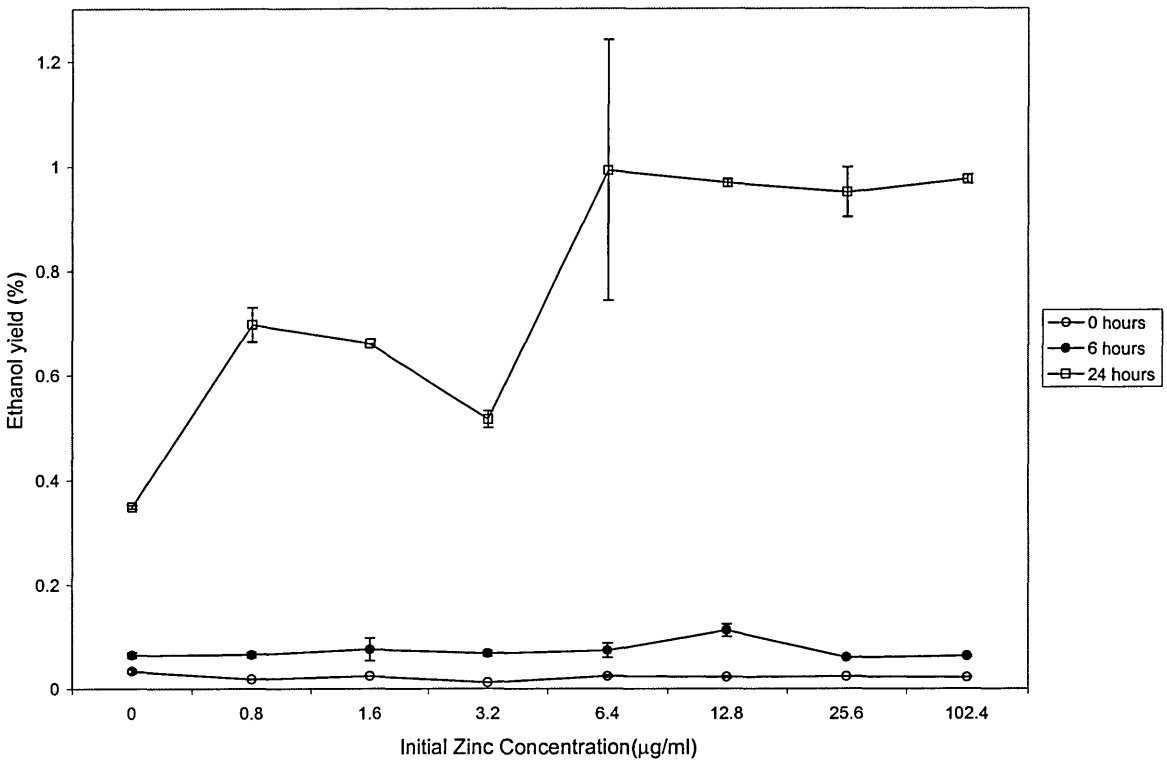
Glucose is the favourite carbon source of *S. cerevisiae* and the preferred mode of metabolism is fermentation. However, the ethanol production rates were relatively low, due to low initial sugar concentration (3%). The maximum ethanol produced after a period of 24 hours was by the lager yeast and reached a yield of 0.991%, when the media was supplemented with 6.4μg/ml of zinc. The maximum ethanol yields by the other strains examined after 24 hours were: distillers yeast, 0.956%; wine yeast 0.61%; and the bakers yeast, 0.596% ethanol. There appeared to be no correlation between the production of ethanol and the initial concentration of zinc, as the different zinc levels which stimulated the maximum ethanol yields were; distillers yeast, 0μg zinc/ml; wine yeast, 25.6μg zinc/ml; bakers yeast 0g zinc/ml.

Glucose repression dictates that *S. cerevisiae* assimilates glucose first, irrespective of the oxygen concentration, as the synthesis of enzymes necessary for disaccharide utilisation (maltose and sucrose) were repressed. Therefore, the induction of a catabolite repressive

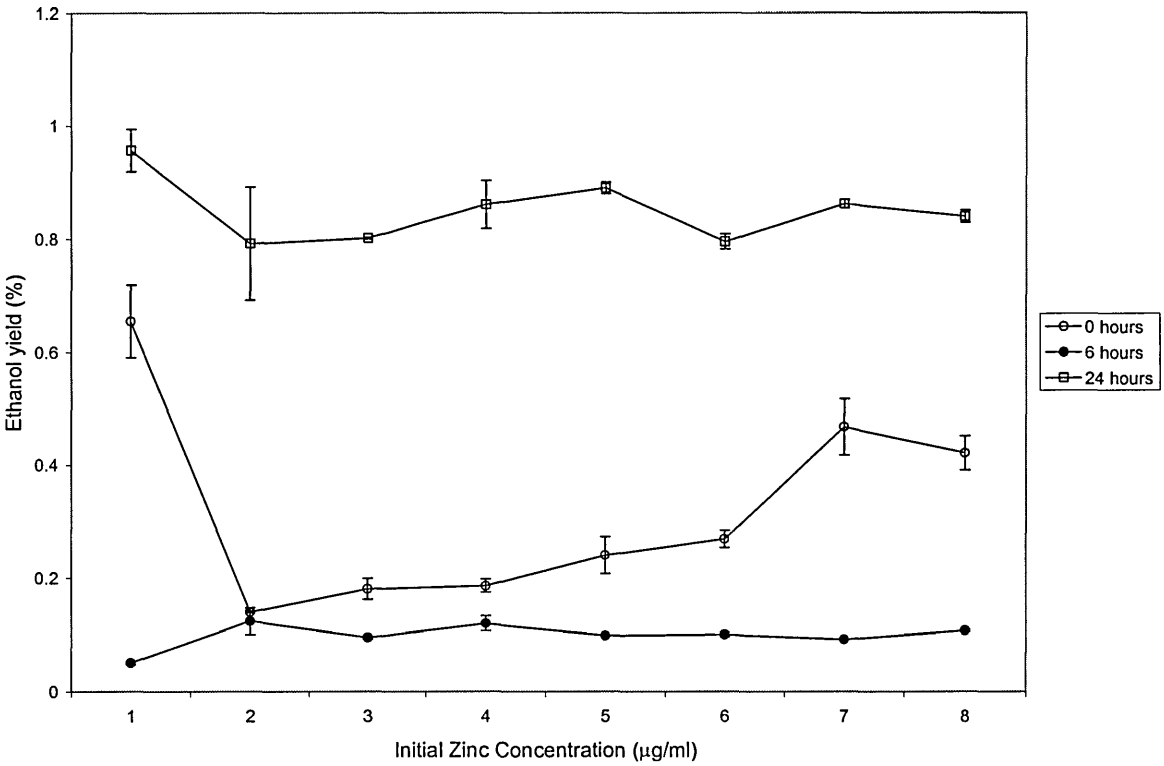
effect is observed in the presence of glucose during mixed substrate (carbon sources) fermentations.

Figure 6.3.6: The effect of zinc on the production of ethanol from glucose (3%) by industrial strains of *S. cerevisiae.*

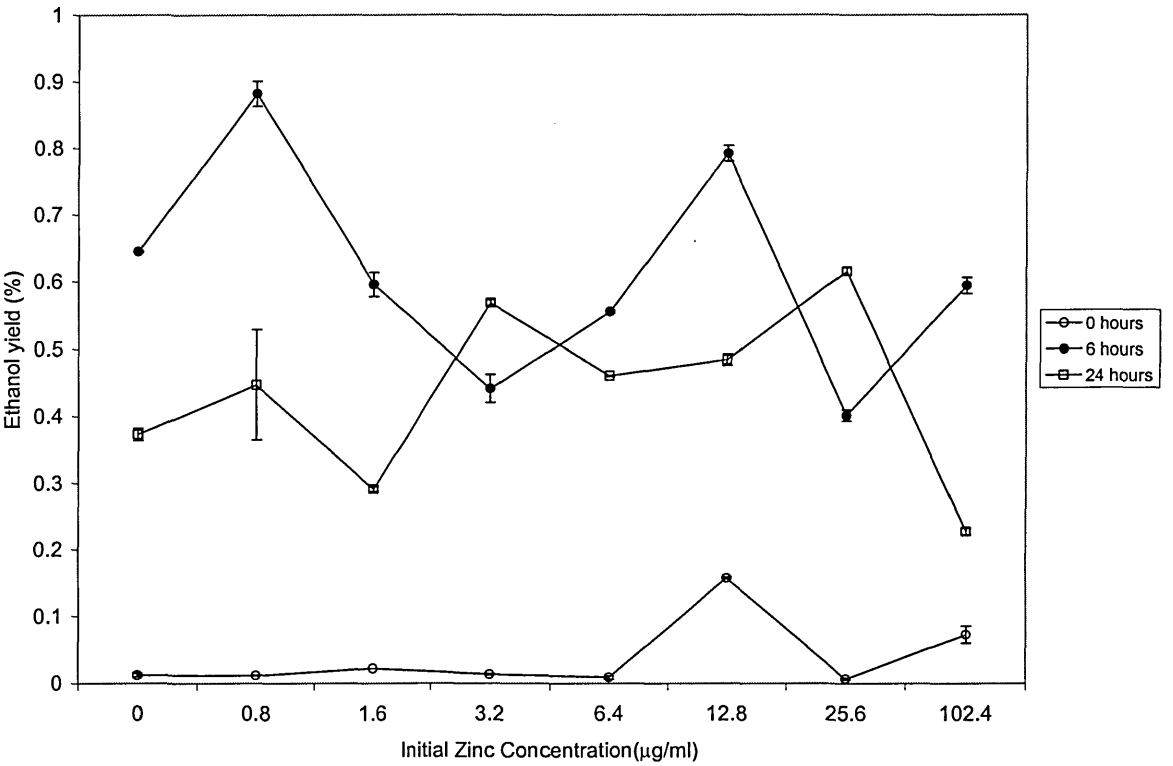
a)Lager yeast



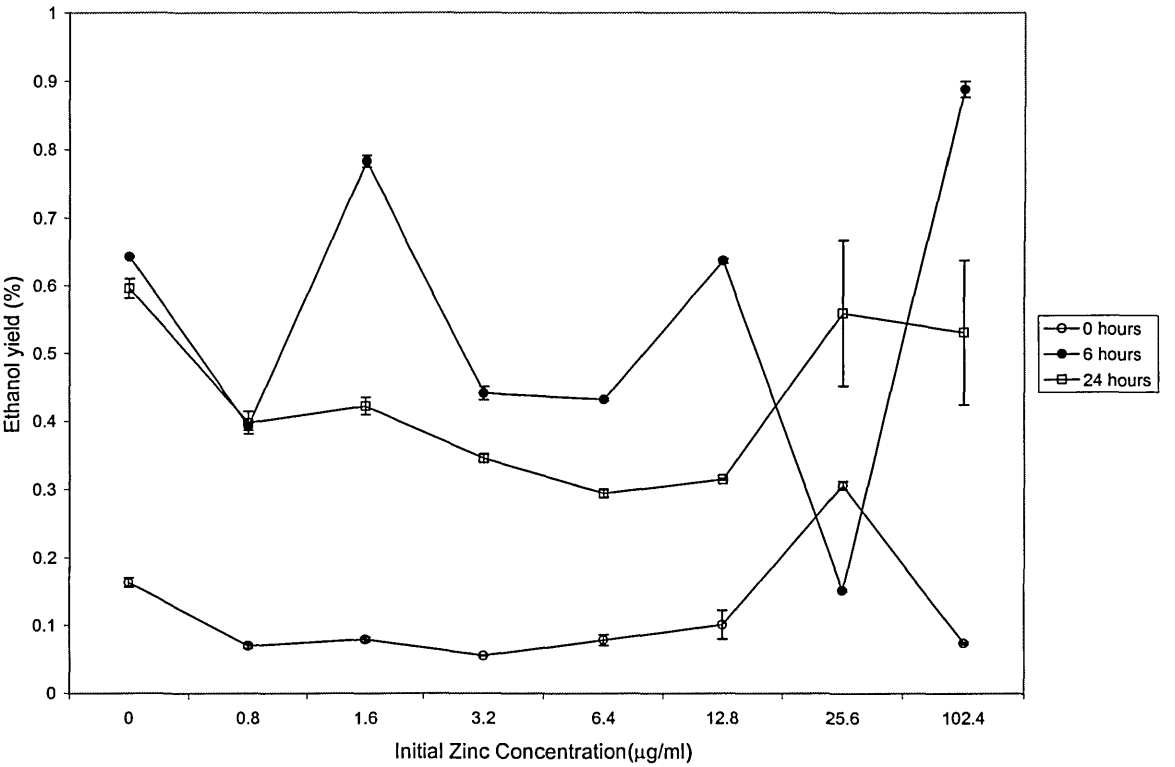
b) Distillers yeast



c) Wine yeast



d) Bakers yeast

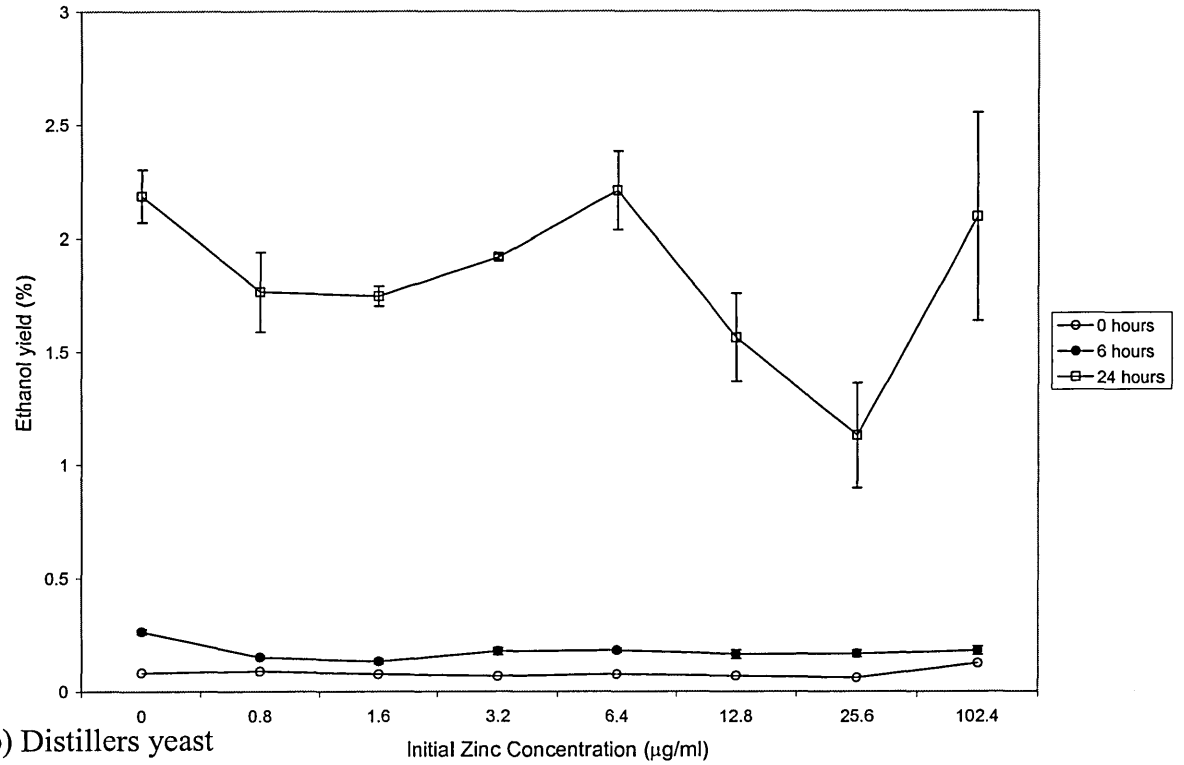


6.3.2.2 Zinc uptake and fructose fermentation by yeast

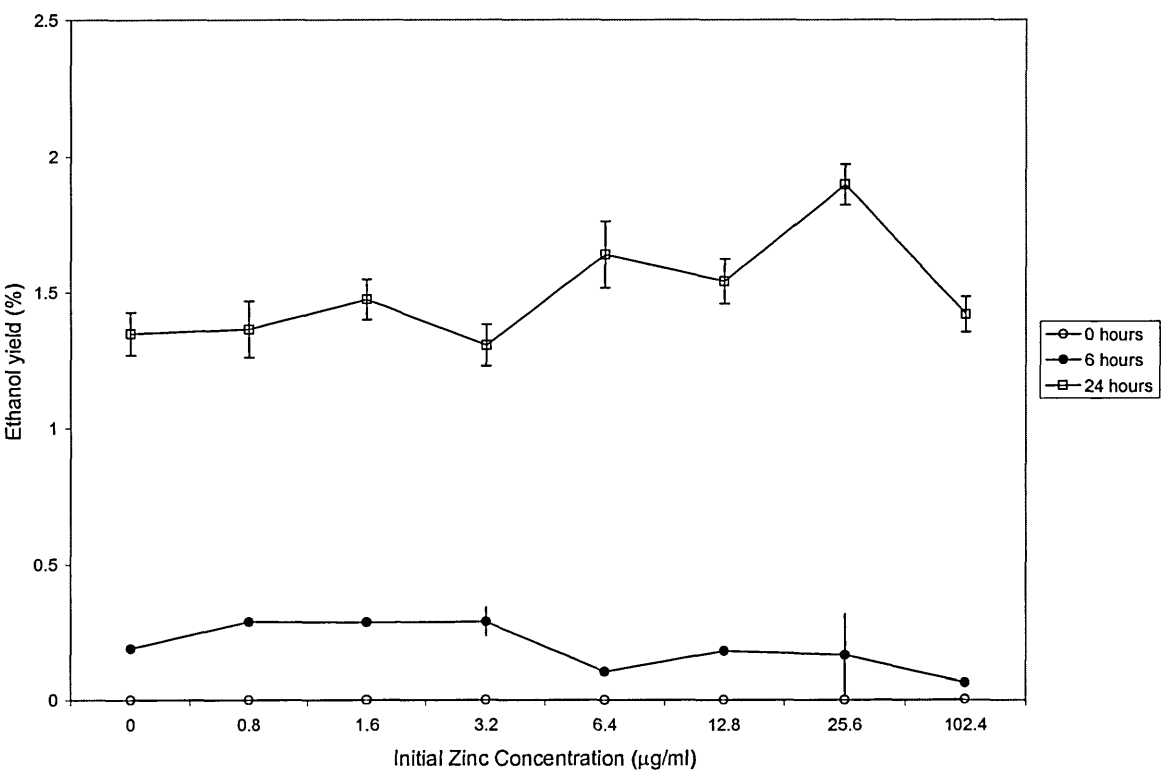
Fructose is transferred into *S. cerevisiae* by facilitated diffusion and enters glycolysis at fructose-6-phosphate stage, where it is catabolised, and in turn produces ethanol. The ability of the industrial strains of *S. cerevisiae* to ferment fructose at different zinc levels was examined and the results are presented in Figures 6.3.7a-d. These results demonstrated a higher ethanol yield over the 24h. than when the yeasts were grown in the YPDM which contained glucose. The maximum ethanol yields are as follows: lager yeast, 2.2%; distillers yeast, 1.897%; wine yeast 1.494% and bakers yeast, 0.776%, when the media was supplemented with 6.4, 25.6, 1.6 and 25.6μg/ml of zinc, respectively. The influence of zinc on the individual strains was stimulatory and the increasing concentrations demonstrating an intra-strain difference in the affinity for the divalent cation which may account for the resultant ethanol yields.

Figure 6.3.7: The effect of zinc on the production of ethanol from fructose (3%) by industrial strains of *S. cerevisiae*.

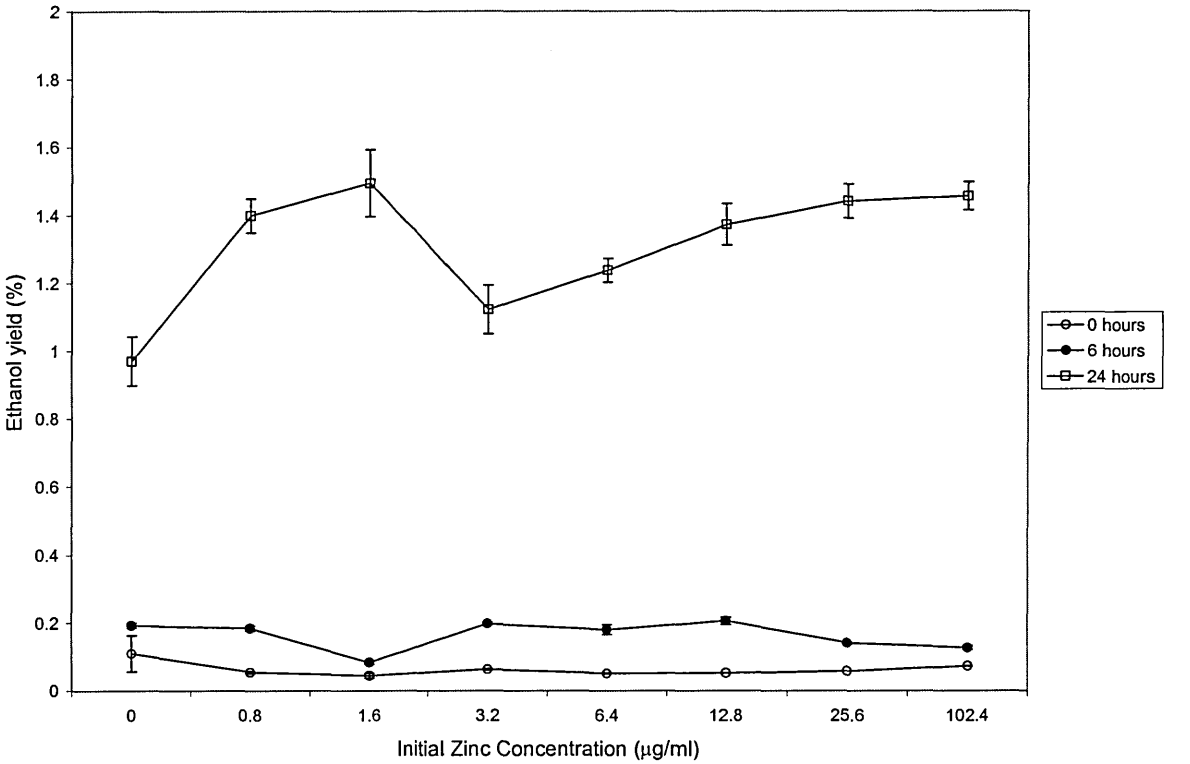
a) Lager yeast



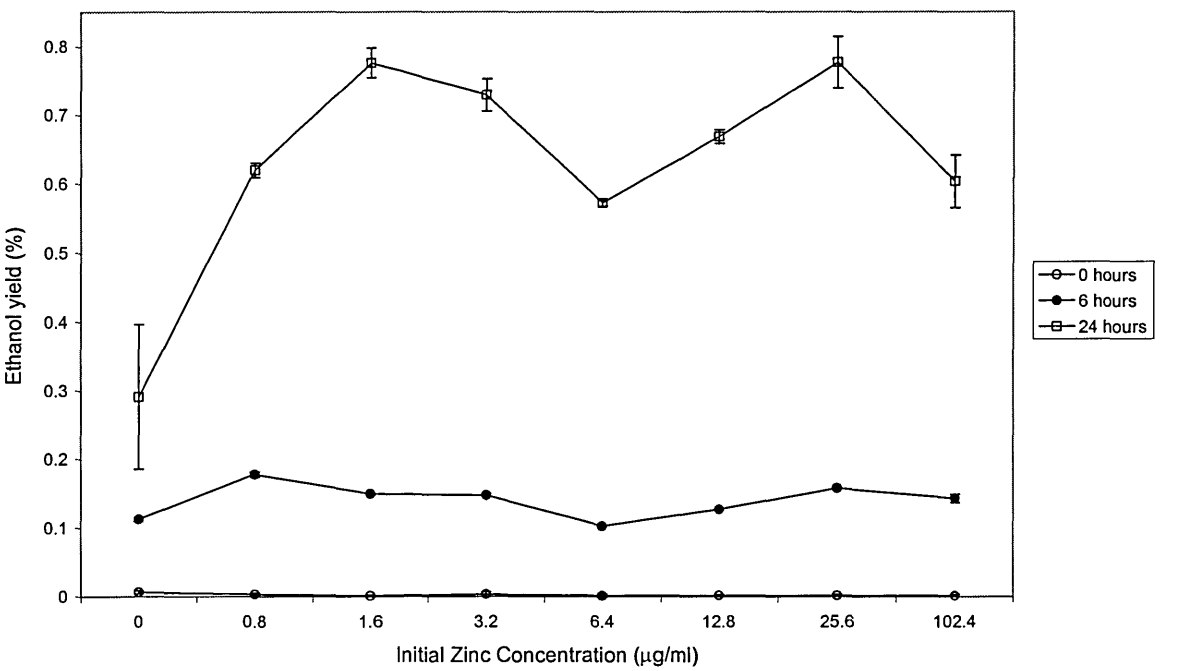
b) Distillers yeast



c) Wine yeast



d) Bakers yeast

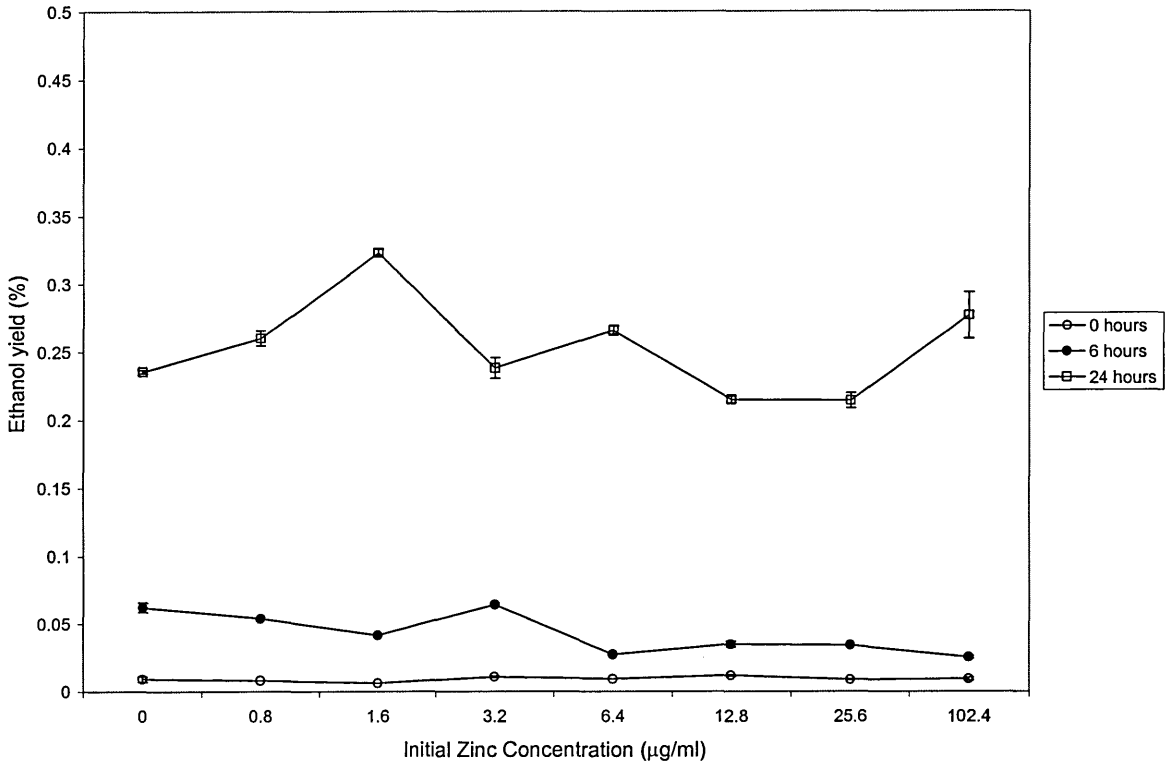


6.3.2.3 Zinc uptake and maltose fermentation by yeast

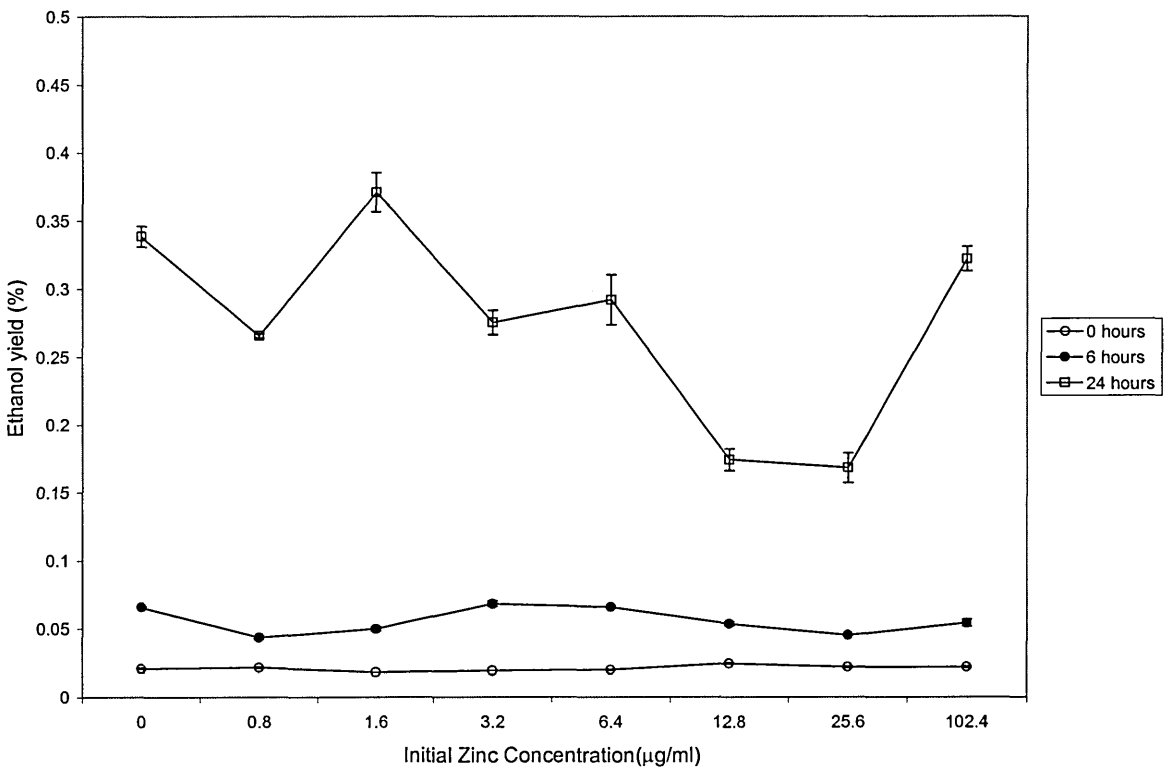
Maltose is utilised by *S. cerevisiae* and its uptake into the cell is controlled by the *MAL* genes (Lagunas, 1993, Dickinson, 1999). Maltose is cleaved intracellularly, by maltase, to the constitutive glucose subunits, whereby energy is produced through glycolysis, and finally ethanol is produced by the cells. Compared with the other sugars maltose was poorly fermented (Figures 6.3.8 a-d), by all the yeast species examined. The lager yeast when grown in the presence of fructose produced approximately 80% more ethanol over the same time period. The maximum ethanol yields after 24 hours were: lager, 0.322%; distillers, 0.37%; wine, 0.359% and bakers, 0.431% ethanol. Under the experimental conditions, bakers yeast produced the most ethanol. The individual species all have different "favourable" substrates, and concomitant differences in the ethanol production rates. The initial zinc concentrations which stimulated these ethanol yields were: 1.6 μg zinc/ml (lager), 1.6 μg zinc/ml (distillers), 0.8μg zinc/ml (wine) and 12.8 μg zinc/ml (bakers). The distillers and the brewers yeast which ferment malt based carbohydrates produced less ethanol than expected, which may be due to the low initial sugar concentration and the unrealistic experimental time, as industrial fermentations using these species would be time, temperature and sugar concentration dependent. The initial zinc concentrations which stimulated these ethanol production rates were generally at the low end of the concentration gradient studied, with the exception of the bakers yeast, which produced most ethanol when the cells were supplied with 12.8 μg zinc/ml in the YPDM, suggesting that these yeasts can still produce high ethanol yields in zinc deficient media.

Figure 6.3.8: The effect of zinc on the production of ethanol from maltose (3%) by industrial strains of *S. cerevisiae*.

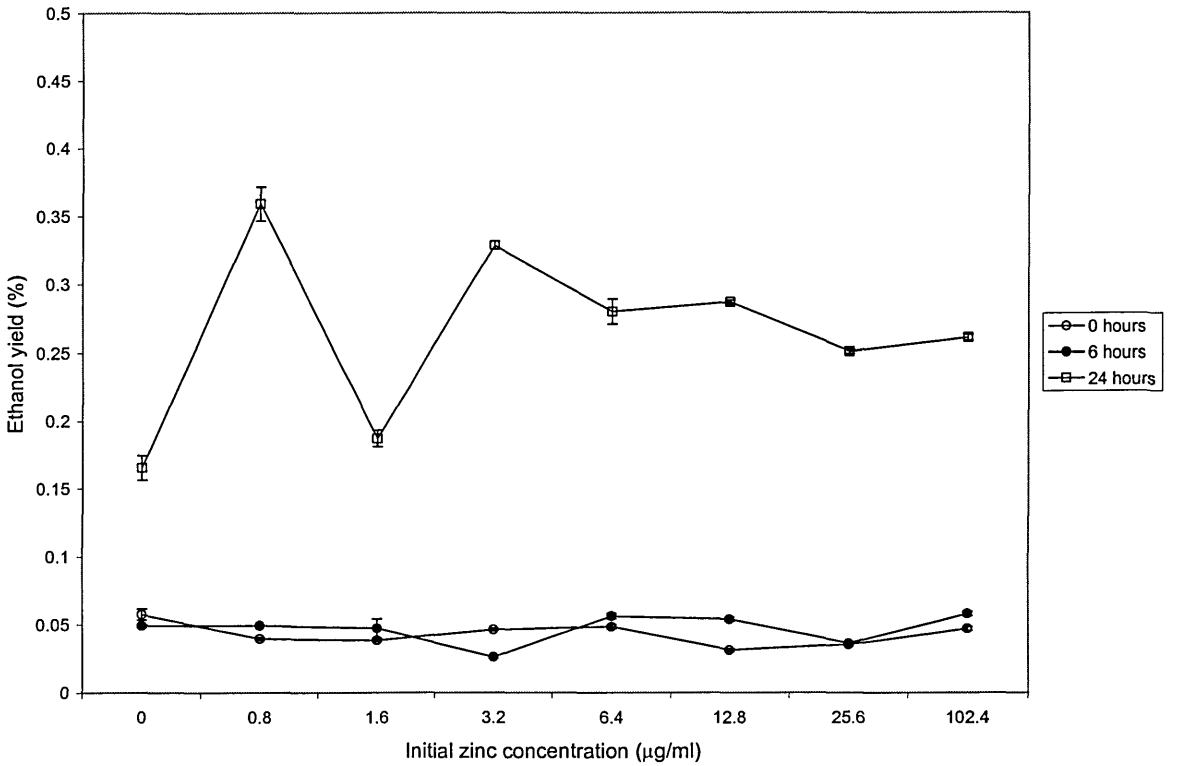
a) Lager yeast



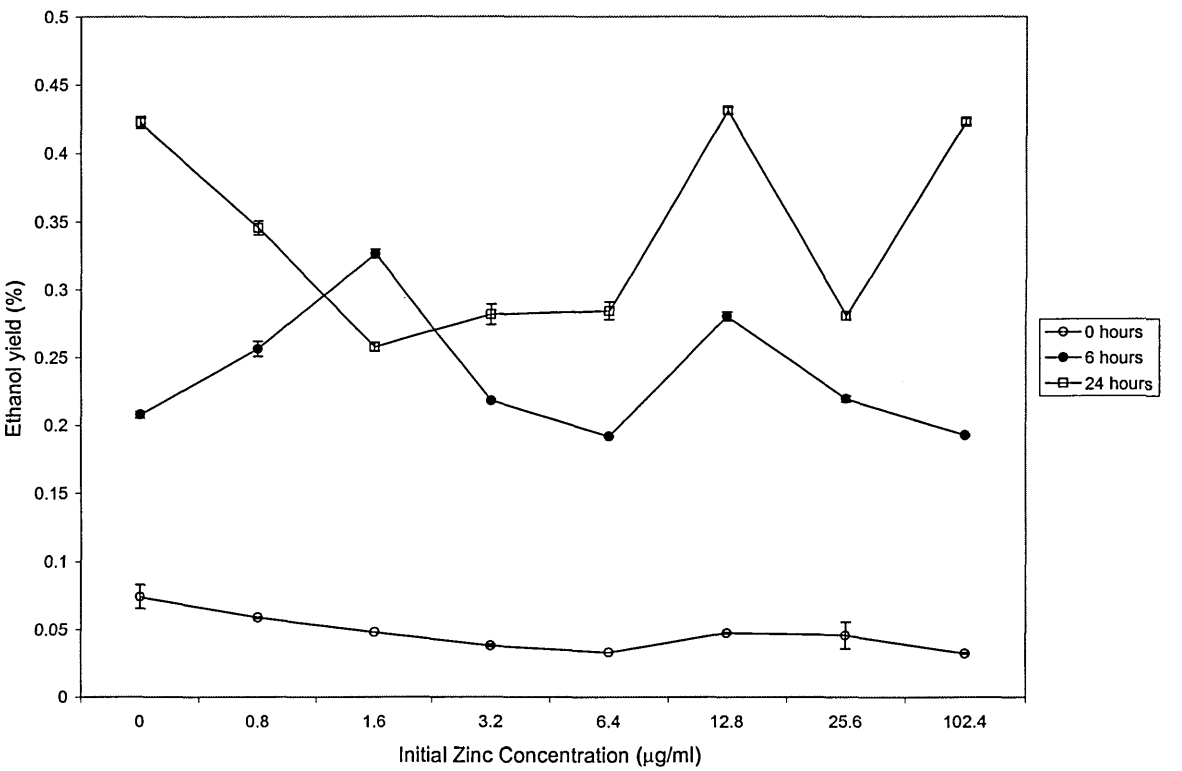
b) Distillers yeast



c) Wine yeast



d) Bakers yeast



6.3.2.4 Zinc uptake and sucrose fermentation by yeast

The disaccharide sucrose is, generally, cleaved extracellularly by invertase, into glucose and fructose. As glucose exerts a repressive effect in response to the presence of other sugars, it was utilised first, followed by fructose. The ethanol production rates of the yeasts when grown in the defined media containing sucrose as the metabolisable energy source (Figures 6.3.10 a-d) were greater than with the disaccharide maltose. Ethanol production rates for the individual species were: lager, 0.823%; distillers, 0.918%; wine, 0.357% and bakers, 0.968%. The levels of zinc which stimulated these ethanol yields were: 0.8 μg zinc/ml (lager), 0 μg zinc/ml (distillers), 0μg zinc/ml (wine) and 1.6 μg zinc/ml (bakers). In comparison to the ethanol production rates of the individual sugars (see figure 6.3.9), the individual monosaccharides produced more ethanol.

Figure 6.3.9: Comparison of the maximum ethanol yields when the industrial yeasts were fermenting different carbon sources with the subsequent initial zinc concentration (expressed in μg zinc/ml) which stimulated these ethanol yields in parenthesis.

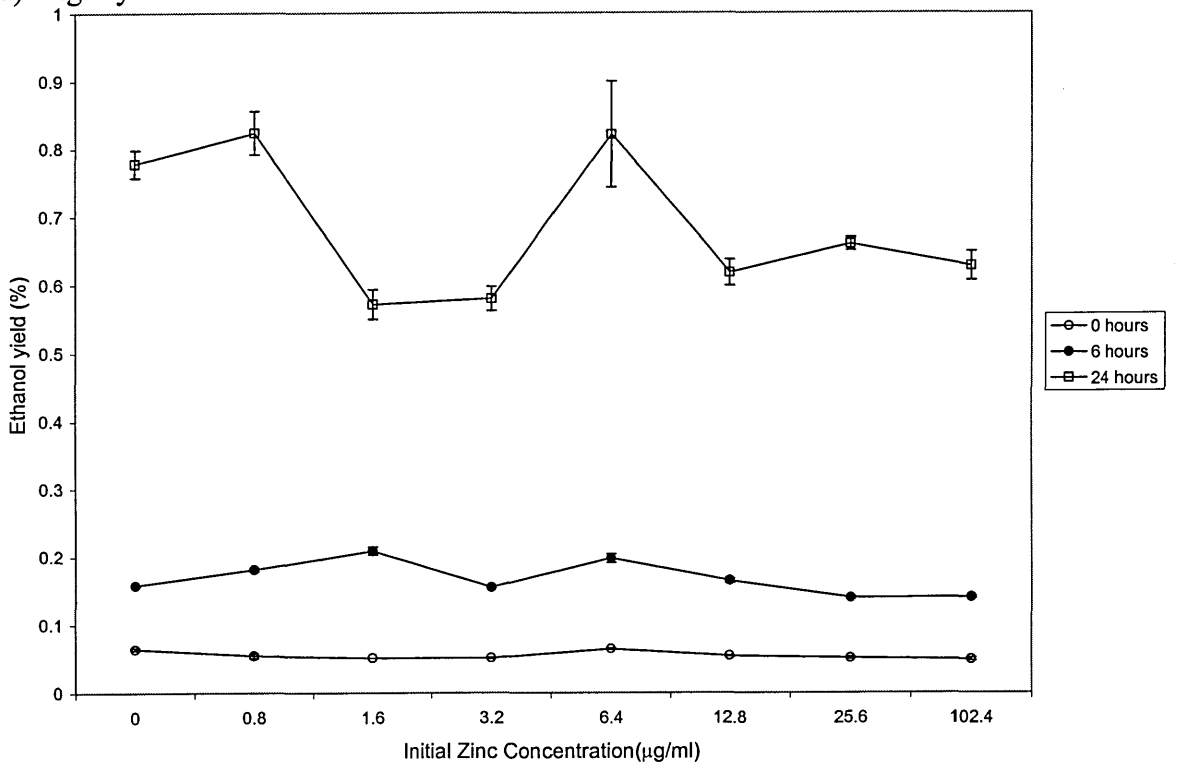
	Ethanol (%)			
	Glucose	Fructose	Maltose	Sucrose
Lager	0.991 (6.4)	2.20 (6.4)	0.322 (1.6)	0.821 (0.8)
Distillers	0.906 (0)	1.897 (25.6)	0.37 (1.6)	0.918 (0)
Wine	0.61 (25.6)	1.494 (1.6)	0.359 (0.8)	0.357 (0)
Bakers	0.596 (0)	0.776 (25.6)	0.431 (12.8)	0.968 (1.6)

With the exception of the bakers yeast, generally more ethanol was produced when the yeasts were supplied with the fermentable monosaccharides (glucose and fructose) than the

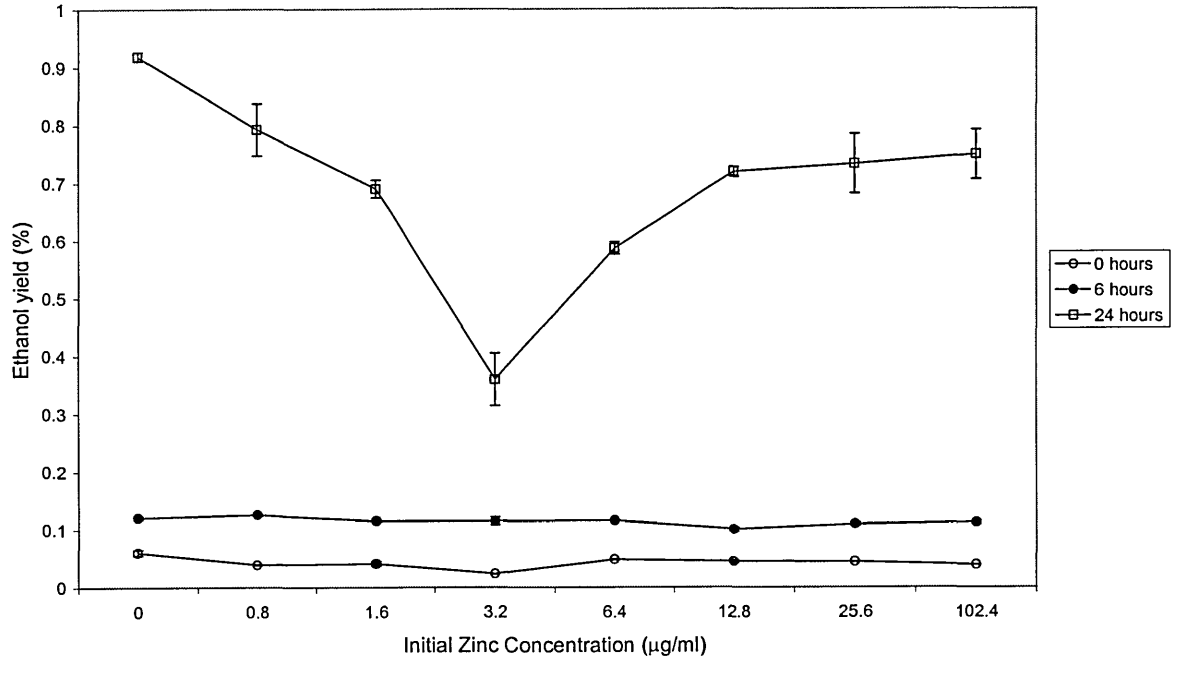
disaccharide (sucrose). With the lager yeast producing the most ethanol when fermenting fructose.

Figure 6.3.10: The effect of zinc on the production of ethanol from sucrose (3%)by industrial strains of *S. cerevisiae.*

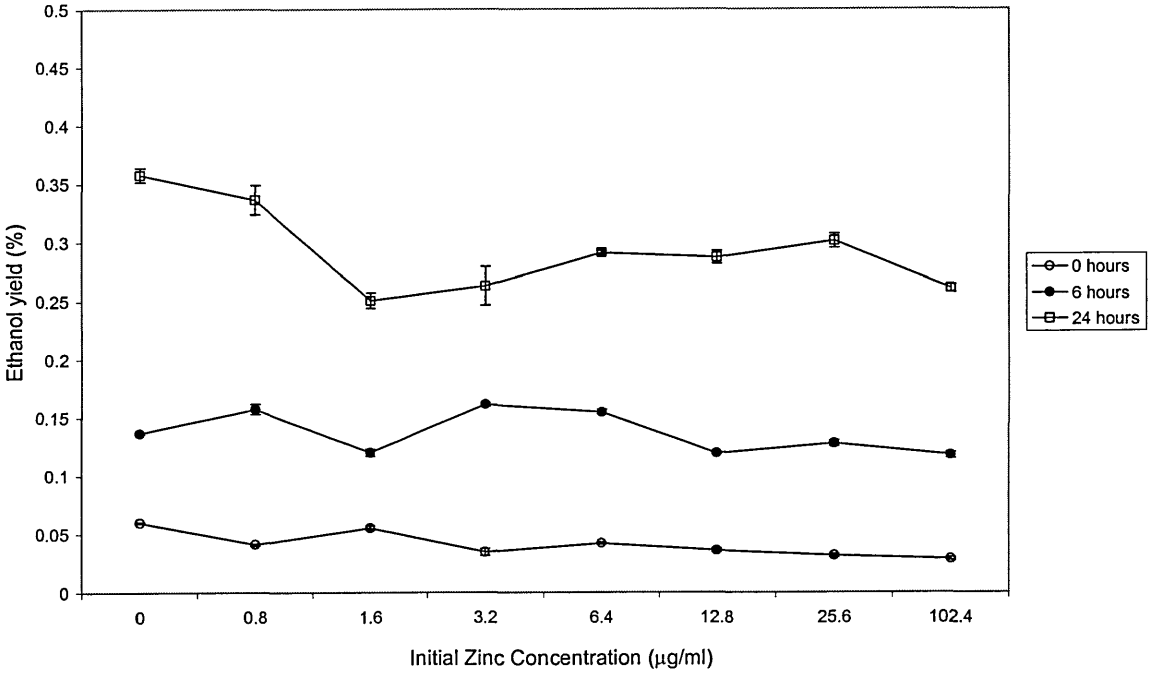
a) Lager yeast



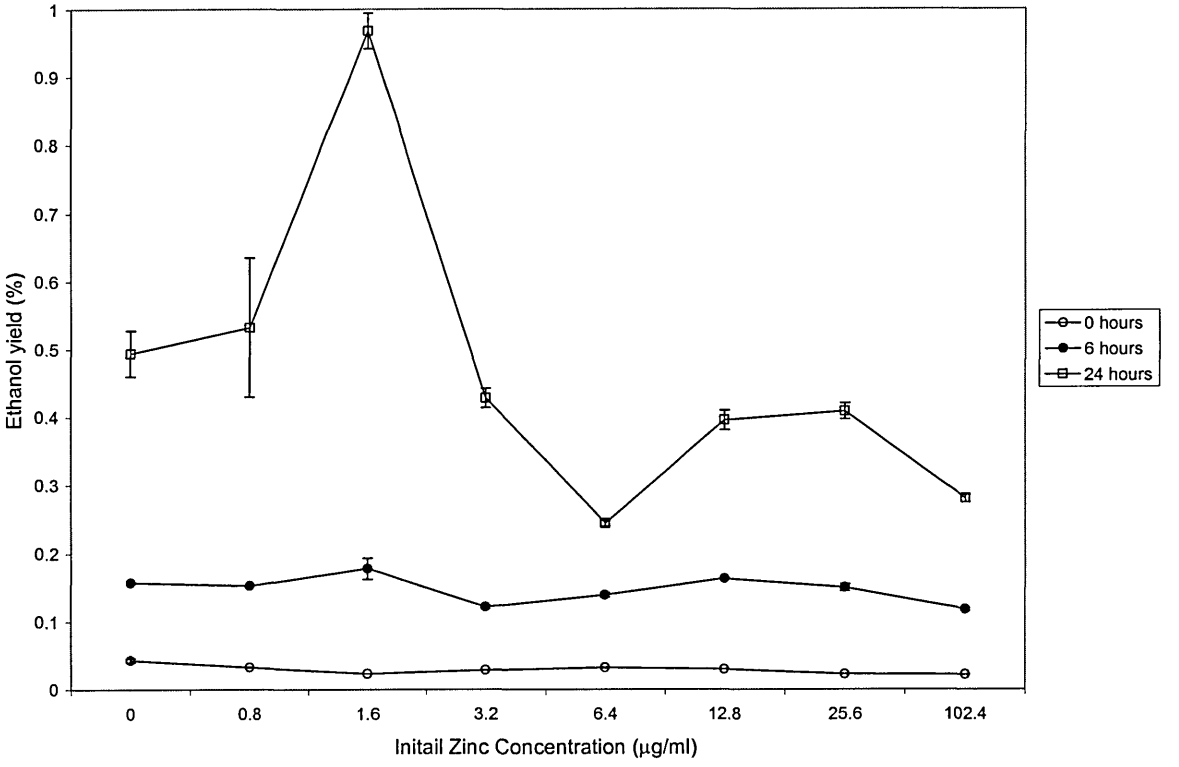
b) Distillers yeast



c) Wine yeast



d) Bakers yeast



From the results (Figures 6.3.6-6.3.10 a-d) it was apparent that the metabolisable energy source influenced the overall ethanol yields. When comparing ethanol producing capabilities of the strains, the lager yeast generally produced more alcohol in media supplemented with 6.4 μg/ml zinc (when the yeast was fermenting monosaccharides). The maximum ethanol produced was when the lager yeast was grown in the presence of fructose, yielding 2.2% ethanol over the 24h. period studied (with glucose yielding 0.991%; maltose, 0.276% and sucrose 0.821%). The concentration of essential metal ions in brewers wort is ever-changing. This natural substrate is affected by many environmental conditions, such as temperature, soil condition *etc.* Studies by Bromberg *et. al.* (1997) on the mineral composition of brewers wort demonstrated an annual variance (between 1994 and 1995) (see Figure 6.3.11).

Figure 6.3.11: Study into the trace metal content of brewers wort (adapted from Bromberg *et. al.*, 1997)

	1994 Malt (mg/l)	1995 Malt (mg/l)
Calcium	35	53
Magnesium	72	86
Mg^{2+} to Ca^{2+} ratio	2.1	1.6
Manganese	0.06	0.14
Zinc	0.02	0.05

Zinc is the only cation that is deficient in wort and is always completely removed by the yeasts (Taidi *et. al.*, 2000) and this figure (6.3.11) demonstrated that brewers wort was deficient in zinc. Bromberg *et. al.* showed that the 1995 malt reached end of fermentation quicker than the 1994 malt. Similarly Figure 6.3.6a demonstrated, enhanced ethanol production with 6.4 μg/ml zinc. It has been shown previously that excessive zinc can inhibit the fermentation performance of brewing yeast (Mochaba *et. al.*, 1996), and the glycolytic enzymes optima for zinc is 15-30μM (Jones and Greenfield, 1984). Therefore, the presence of zinc is influential in ethanol production by industrial strains of *S. cerevisiae.* The distillers yeast was influenced by the parameters mentioned above (deficient ion content of natural substrates and annual variance of the substrate). Distillers yeast, like lager yeast, fermented fructose most efficiently, and achieved a maximum ethanol yield of 1.897%, in comparison to 0.956% (glucose), 0.37% (maltose) and 0.918% (sucrose). The initial zinc concentrations that stimulated this ethanol production did not fall into a clear pattern of stimulation (like the lager yeast). However, if the fermentation was permitted to continue, a relationship between, zinc concentration and ethanol production might have been established.

Maximum ethanol production by the wine yeast was also achieved using fructose as the energy source. Fructose, a constituent of grape must, allowed the wine yeast to produce the most ethanol (1.494%) over 24hours The amount of ethanol that was produced when fructose was the supplied carbohydrate, was much greater than when the other carbohydrates were supplied (0.61%, glucose; 0.359%, maltose and 0.357%, sucrose). The levels of zinc that stimulated the ethanol production were 1.6, 25.6, 0.8 and 0μg/ml, fructose, glucose, maltose and sucrose, respectively). The variable of temperature on winemaking has the ability to significantly affect the growth of the yeasts, therefore the overall fermentation performance (Charoenchai *et. al.*, 1998)

The ethanol production rates of bakers yeast were maximal when the supplied sugar was sucrose, with an ethanol yield of 0.968%. The ethanol production rates of the bakers yeast when grown in the other carbohydrates was 0.596% (glucose); 0.776% (fructose) and 0.431% (maltose). There was no obvious pattern linking available zinc concentration and ethanol output.

6.4 Conclusion

In conclusion, the experimental aims of this Chapter were met, and the influence of zinc on yeast physiology examined. Zinc stimulated ADH activities with maximum ADH activity observed when media was initially supplemented with 12.8μg/ml zinc. The increased activities of this intracellular enzyme generally increased the ethanol output after 24 hours. In further experiments, the influence of both initial zinc concentration and metabolisable energy source present was examined with respect to final ethanol yields. These final ethanol yields were optimal when the lager yeast was grown in a minimal media containing the monosaccharides, glucose or fructose, with an initial concentration of zinc at 6.4 μg/ml. This result established that zinc does have a positive effect on the ability of lager strains of *S. cerevisiae* to produce ethanol, as does the presence and type of metabolisable energy source. Fructose stimulated the greatest ethanol yield, and this resulted in a 2.2% yield after a period of 24 hours.

Chapter 7

Concluding Discussion

The ability of *S. cerevisiae* to sequester heavy metals is known to be a biphasic response consisting of a metabolism independent and a metabolism dependent stage (Gadd, 1993). Recent research has established the presence of 2 plasma membrane transporter proteins (*ZRT1 and ZRT2)* which operate under zinc limiting and zinc replete conditions, respectively (Zhao and Eide, 1996 a & b). These transporters allow for the biological accumulation of zinc. Once the zinc has entered the cell, it can either be used immediately or stored under zinc replete conditions until required.

The aims of the research were to determine the effect that zinc has on certain aspects of yeast cell physiology and also the effect of yeast cell physiology on zinc uptake and accumulation, using industrial relevant strains of *Saccharomyces cerevisiae.*

The experimental approach adopted was simple, and used basic laboratory equipment and techniques throughout the majority of the research, involving cell number determination, viability assessments and atomic absorption spectroscopy. These techniques were employed in order to determine the effect of media supplemented with zinc on yeast cell growth and metabolic activity. Other aspects of the work focussed on, zinc uptake into the viable cell, the parameters which affected the uptake, and if zinc could enhance alcohol dehydrogenase activity and ethanol production. In addition, protective effects of zinc on stressed cells was investigated.

The experimental approach adopted allowed the research to determine if zinc was influential in matters pertaining to yeast cell physiology and if yeast cell physiology could influence the degree of zinc uptake and accumulation within *S. cerevisiae.*

The main findings of the research were that:

- Zinc uptake was instantaneous and removal of the zinc from the cell wall material using general and specific chelating agents demonstrated that in this instance zinc uptake was not reversible.
- Once the zinc was taken up into the cell, it is either utilised immediately or stored until the nutritional sensing within the cell dictated zinc-limiting conditions, as the level of free zinc within the viable "Servomyces" product was minimal. Non-viable yeast preparations, on the other hand which were capable of removing zinc from the environment, localised the zinc ions in the cell wall.
- Zinc accumulation was influenced by biological, chemical and physical parameters as indicated below:

Factors influencing metal ion accumulation by S. cerevisiae

Biological	Chemical	Physical
Cell Density	Presence of metabolisable energy source	Temperature
Strain of *S. cerevisiae*	Type of metabolisable energy source	pH
Culture Viability		
Physiological state		
Growth phase		

- Zinc accumulation was a cyclical event, with the majority of zinc accumulation occurring during the lag and the early exponential phase.
- Zinc ions appeared to convey a protective effect on lager yeast.
- Increased zinc uptake, increased the level of ADH activity within a brewing lager strain, which in turn, stimulated the cell to produce more ethanol.

The many processes that depend on zinc uptake into the yeast cell would be inadvertently effected if this essential ion was deficient. Zinc uptake into *S. cerevisiae* is a cyclical event, and was associated with the lag and the early exponential phase of the yeast growth cycle. During these stages the cells are adapting to their new environment and entering the cell division cycle of cellular growth and reproduction. During this time the zinc is essential for DNA and RNA replication (with zinc being incorporated into the polymerase enzymes) and also in the stabilisation of these nucleic acids. Zinc ions are to be found in the catalytic site of enzymes and the ions also play a role in the structure and function of many proteins (*e.g.* zinc finger proteins). Another important zinc metalloenzyme is alcohol dehydrogenase. This enzyme is important in the glycolytic pathway, as a means of regenerating NAD from fermentation. The results demonstrated that culturing yeast in different initial concentrations of zinc, could enhance the enzyme activity, which in turn positively influenced the amount of ethanol produced. This cyclical accumulation during the early stages of growth appears to convey a protective effect in cells which have been subjected to a chemical and a physical stress. The speculated role of zinc in the family of proteins which confer stress protection is unconfirmed but possible. Therefore, the pre-conditioning of brewing lager yeast with excess zinc, might improve the viability and the vitality of the yeast, thereby, allowing the cells a degree of protection against the stresses of the fermentation procedure, which may allow the cells to produce more ethanol. For maximum zinc accumulation, a metabolisable energy source must be present. However, the concentration and the chemical basis of the carbohydrate was extremely influential when examining zinc uptake by industrial strains of *S. cerevisiae*. The results demonstrated that monosaccharides generally stimulated greater uptake rates. Perhaps this is due to energetic considerations since, for example, glucose uptake by *S. cerevisiae* is energy independent whilst that of maltose requires ATP. As a consequence less energy would be available for

zinc uptake in the presence of maltose. Another consideration might be that for the utilisation of disaccharides, energy is required for enzyme synthesis which results in hydrolysis of disaccharides. Monosaccharides do not require energy expenditure for the production of such hydrolytic enzymes, therefore, could utilise this conserved energy to sequester zinc. Physical factors also influence the amount of zinc accumulated by *S. cerevisiae*. When the brewing lager yeast was subjected to a temperature stress of 4°C, the zinc accumulation ability of the industrial yeasts was reduced. This was probably due to the changes in the plasma membrane, because at 4°C, the membrane cools and arrangement becomes more crystalline, and so the ability of the trans-membrane proteins to transport zinc into the cell will be affected. Therefore, the overall zinc accumulating ability of the cell will be reduced. During these conformational changes, the cell wall was still capable of sequestering zinc ions.

In conclusion, the results presented in this thesis demonstrated that zinc was accumulated by *S. cerevisiae* in a cyclical manner, and that uptake could be manipulated by the presence and type of carbon source, as well as chemical and physical parameters.

Further work that may be developed from this research are as follows:

- Determination if zinc could alleviate the detrimental effects of stress on yeast cells. This work would not only be physiologically based, but could examine, by the use of electrophoretic techniques, the effects of zinc limiting and zinc replete cultures on the production of stress proteins.

- Examination of the zinc uptake ability and the factors which influence accumulation in other industrial strains of yeast, therefore, determining whether metal ion uptake is universally affected by the biological, chemical and physical parameters that have affected zinc accumulation in *S. cerevisiae*.

- Examination of the use of zinc pre-conditioning in industrial fermentations in order to improve the vitality of the yeasts, resulting in the yeasts being protected from the stressful effects of the fermentation procedure, and possible allowing the yeasts to produce more ethanol.

Chapter 8

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